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ADHESION OF HUMAN MELANOCYTES TO EXTRACELLULAR MATRICES BY INTEGRINS:

EFFECT OF CELL DIFFERENTIATION AND GROWTH ON CELL-SUBSTRATE ADHESION

BY

© **GORDON EDWARD SEARLES**

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

in

Experimental Medicine

DEPARTMENT OF MEDICINE

EDMONTON, ALBERTA

SPRING 1995

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15 November 1994.

TO SUSAN, MY LOVING WIFE, FOR HER UNTIRING PATIENCE AND MORAL SUPPORT FOR ALL THIS TIME; AND TO MY PARENTS, WHO HAVE ALWAYS BELIEVED IN ME.

ABSTRACT

Malignant tumour cells acquire the ability to invade tissues and form secondary metastatic colonies. Malignant cells also appear to express a less differentiated cellular phenotype, compared to normal cells. Studies on malignant melanoma tumour progression suggest that changes in the expression of integrin cell-substrate adhesion molecules may be one of the first altered molecular events in tumourigenesis. In order to define whether integrin expression and function is related to tumour growth and cell differentiation, the integrin expression and function was examined in normal and malignant melanocytes *in vitro* which represented specific growth phases of malignant progression *in vivo*. These included the radial growth phase (RGP), vertical growth phase (VGP), and metastatic growth phase (MGP). Phenotypic expression of major melanocyte differentiation markers on normal and malignant melanocytes obtained from normal human skin and malignant melanoma tumours were first characterized. The surface expression of individual integrin subunits, and the cell-substrate adhesion strength to extracellular matrix substrates were then compared. Finally, perturbation studies affecting adhesion strength were performed using blocking anti-integrin antibodies, and adding extracellular divalent cations to the culture medium, which could alter the conformation of the integrin binding site. All cells derived from each tumour progression phase expressed the same limited number of integrin subunits. The integrin expression levels were greatest for the RGP cells, and progressively declined with advancing tumour progression phase. There were phase-dependent changes in cell adhesion to fibronectin, laminin, and collagens type I and IV. Contrary to expectation, RGP cells had poor adhesion strength to all substrates, while VGP cells had poor adhesion strength to laminin. Adhesion strength could be regained by extracellular manganese, suggesting that the integrins expressed by RGP and VGP cells are in a reversible non-active state. In order to examine whether cell differentiation was related to integrin function, cells from each tumour progression phase were exposed to the differentiation-inducing agents, bromodeoxyuridine (BRDU) and α-melanocyte stimulating hormone (α-MSH). BRDU exposure produced a less differentiated cell type, while α-MSH exposure produced a more differentiated cell type. The integrin expression and function were also changed such that the characteristics became similar to the more or less advanced tumour progression phase, respectively.

This study showed that (1) melanoma cells became less differentiated with advancing tumour progression phase; (2) melanoma cell differentiation could be influenced by BRDU and α-MSH; (3) while the repertoire of integrin subunit expression was more or less the same during tumour progression, the quantitative levels were different and greatest in RGP cells; (4) integrin expression and function are related to the cellular differentiation state; and (5) RGP cells expressed integrins

reversibly into a non-active form. The results clearly demonstrate that the cell surface expression and function of integrins are related to the growth and differentiation of human melanocytes in both normal and neoplastic conditions.

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LIST OF ABBREVIATIONS

BRDU:	5-bromo-2'-deoxyuridine
CI:	Collagen Type I
CIV:	Collagen Type IV
COLL:	Collagen
DPhe:	D-phenylalanine
DMSO:	Dimethyl sulfoxide
DOC:	Deoxycholate
EDTA:	Ethylene diamine tetraacetic acid
FBS:	Fetal bovine serum
FN:	Fibronectin
GRGDS:	Glycine-Arginine-Glycine-Aspartic Acid-Serine
GRGES:	Glycine-Arginine-Glycine-Glutamic Acid-Serine
L-DOPA:	L-deoxyphenylalanine
LN:	Laminin
α-MSH:	α-melanocyte stimulating hormone
MGP:	Metastatic growth phase
N:	Normal growth phase
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
Nle:	Norleucine
NP-40:	Nonidet P-40
PdBU:	Phorbol-12,13-dibutyrate
Phe:	Phenylalanine
PPO:	Diphenyloxazole
RGP:	Radial growth phase
RIPA:	Radio-immunoprecipitation assay
RPMI:	Roswell Park Memorial Institute
SDS-PAGE:	Sodium docedyl sulfate-polyacrylamide gel electrophoresis
VGP:	Vertical growth phase

I. INTRODUCTION: EXPRESSION OF CELL-SUBSTRATE ADHESION AND DIFFERENTIATION PHENOTYPE IN HUMAN MELANOCYTES AND THEIR MALIGNANT PROGRESSION

OVERVIEW

During malignant tumour progression cells acquire the ability to invade tissues and form secondary metastatic colonies. Malignant cells also appear to express a less differentiated cellular phenotype, compared to normal cells. Studies on malignant tumour progression in melanoma suggest that the expression of cell-substrate adhesion molecules, mediated through integrins, is one of the first molecular events to alter. In order to define the relationship between integrin expression and cell differentiation and growth, the expression and function of integrin cell-substrate adhesion molecules will be examined in normal and malignant melanocytes *in vitro*. The normal and malignant melanocytes will be obtained from normal human skin and malignant melanoma tumours representing specific growth phases of malignant progression *in vivo*. The cell lines will be first characterized by their phenotypic expression of major melanocyte differentiation markers. The phenotypic expression will be compared with surface expression of individual integrin subunits, and cell-substrate adhesion to extracellular matrix substrates. Perturbation studies will be performed using blocking anti-integrin antibodies, and adding extracellular divalent cations to the culture medium. Cells with identical genotype, but expressing different phenotypes, will be created by using the differentiation-inducing agents, bromodeoxyuridine (BRDU) and α-melanocyte stimulating hormone (α-MSH). Integrin expression and function will be compared among these cell lines derived from specific tumour progression phases. The outcome of these studies would determine how the cell surface expression and/or the function of integrins might be related to the growth and differentiation of human melanocytes in both normal and neoplastic conditions.

BIOLOGY OF MELANOCYTE DIFFERENTIATION

Embryologic Differentiation of Melanocytes

Epidermal melanocytes are derived from the neural-crest tissue, and migrate to the epidermis very early in embryonic development [Rawles, 1948]. These dorsal neural crest-derived cells comprise the melanocytes found in the skin and mucous membrane, as well as the eye and central nervous system. The first precursor cells can be identified in the skin by about eight weeks of gestation [Sagebiel and Odland, 1972]. These precursor cells have measurable tyrosinase activity in the cytoplasm, and express several antigens which are thought to be unique to melanocytes (HMB-45, CF21, TRP-2) [Bennett, 1993; Lynch et al, 1991] but do not yet contain functional melanosomes or form the functional interactions with surrounding cells.

The differentiation of melanoblasts into melanocytes is dependent not only on the genetic information supplied by melanoblasts, but also on the interaction with the surrounding cells. Weiss and Andes [1952] injected melanoblasts from one chick breed into the circulatory sytem of another chick breed, and found that the injected melanoblasts acquired characteristics of the melanocytes found in the host chick breed. Thus, the local environment, in particular the functional interaction with neighbouring cells, can have a profound influence on the expression of differentiated phenotypes which are genetically preprogrammed by the developing melanoblasts.

The average number of melanocytes per unit area is constant between various skin colors. However, the melanocyte density appears to increase with stimulation by wounding [Potten et al, 1971], or by exposure to ultraviolet radiation [Quevado et al, 1965]. Accordingly, there is a low level of melanocyte turnover in non-irradiated skin, suggesting a small degree of mitotic capability, despite their fully differentiated phenotype. Thus, the presence of such developmental features as melanosomes and tyrosinase activity does not interfere with mitosis, and, unlike their counterparts in the central nervous system, retain their capacity for division in the post embryonic period [Jimbow et al, 1975].

Function of Epidermal Melanocytes

Epidermal melanocytes are the major cells responsible for skin colouring of the host. This pigmentation is divided into two forms: constitutive skin color, and facultative (or inducible) skin colour. The constitutive skin colour is the amount of cutaneous melanin pigmentation generated according to cellular genetic programs without any direct effect by any radiations of solar origin [Jimbow et al, 1993]. Facultative skin color can be exemplified by the tanning reaction that occurs following direct exposure to ultraviolet radiation, or from hormonal influences [Jimbow et al, 1993].

One of the major functions of human melanocytes is the skin protection against the damaging effects of ultraviolet radiation. The energy spectrum contained within the ultraviolet A (UVA, 320 - 400 nm), and ultraviolet B (UVB, 280 - 320 nm) is capable of forming highly reactive oxygen species through free radical formation, which would react with and intercalate sensitive cellular enzymes and nucleic acids. The loss of this melanocyte function is highlighted by medical conditions seen in patients suffering from cutaneous albinism, in which the melanocytes are nonfunctional, and hence no melanin pigments are present in the skin. Those individuals whose natural skin colour is white, are extremely sensitive to even small exposures to ultraviolet radiation. They also have a much greater risk of developing cutaneous malignancies, such as basal cell and squamous cell carcinomas. Conversely, such malignancies are rare in dark-skinned people, and almost nonexistent in black-skinned races, supporting the photo-protective role of melanin pigments in the skin.

The photoprotective function is performed by the energy-absorbing characteristics of the melanosome, which is a secretory intracellular organelle of the melanocyte. The melanosome is composed of four elements: structural proteins, tyrosinase, outer membrane and certain auxiliary enzymes [Jimbow et al, 1976]. The structural components are constructed following Golgi-associated processing to form a matrix of concentric sheets [Birbeck and Barnicot, 1959; Jimbow and Kukita, 1971; Schroeder, 1969]. The addition of tyrosinase is thought to occur either during or shortly after the construction of the matrix within a membrane-bound organelle. While several hypotheses exist regarding how tyrosinase is incorporated within the melanosome [Birbeck and Barnicot, 1959; Jimbow et al, 1971; Wellings and Siegel, 1963; Maul, 1969; Novikoff et al, 1968], the current hypothesis describes a specialized organelle called the vesiculoglobular body [Maul, 1969; Maul and Brumbaugh, 1971; Novikoff et al, 1968], which arises from the Golgi apparatus separate from the structural proteins. These vesiculoglobular bodies are transferred into the vesicles containing the structural proteins, where the tyrosinase is deposited onto the matrix. This matrix supposedly will hold the tyrosinase in an optimal conformation, permitting melanization to proceed at a rapid pace.

Each of the melanosomal components are under genetic control, with the key regulatory steps associated with the transcription and translation of information being coded by pigmentary genes [Stern, 1970]. The level of control is exerted at both the transcriptional and the translational levels, to control the number and extent of melanosomal structure [Jimbow et al, 1993]. Genetic studies of mammalian systems have demonstrated an inverse relation between melanization and mitosis. Retinal pigment epithelium cells of mice do not form melanosomes during division, only to rapidly produce melanosomes once division has stopped [Whittaker, 1963 and 1965]. Conversely, hormones such as α-melanocyte stimulating hormone (α-MSH) increase melanization of melanocytes, without any effect on mitosis [Lerner, 1971].

Epidermal-Melanin Unit

Four factors were found to be critical controlling agents regarding melanocyte differentiation and function: (1) the genotype of the melanoblast, (2) the genotype of the environmental cells, (3) the environmental history of the melanocyte, and (4) the characteristics of the differentiated environmental cells [Markert and Silvers, 1956]. Each of these components can be conceptually linked through a functional, synergistic unit whereby a normal melanocyte interacts with the surrounding keratinocytes and the underlying dermis. This concept was first proposed by Breathnach and Fitzpatrick [1963], and was called the epidermal melanin unit (EMU). The number of active EMU varies markedly between different regional sites in the skin [Szabo, 1967], while the ratio of keratinocytes to melanocytes within epidermal melanin units, however, remains constant [Frenk and Shellhorn, 1969].

Local factors can also regulate the rate of mitosis by melanocytes. While multiple theories have been put forth to explain the nature of this regulation [reviewed in Jimbow et al, 1976], it is widely held that changes in the local environment will alter the function of the EMU in such a manner that would attempt to restore the previous equilibrium. This regulation may use soluble or contact signals produced by keratinocytes, which are received by the melanocyte. However, the nature and control of these factors is still not known.

Melanocyte Differentiation and Melanogenesis

The key events in the formation of skin colour include: (1) the migration of the melanoblasts from the embryonic neural crest tissue and their differentiation to form epidermal melanocytes in the skin; (2) the formation of structural proteins and the enzyme tyrosinase and their assembly in the development of melanosomes within the melanocyte; (3) the melanization of melanosomes; (4) the movement of melanosomes from the perikaryon to the dendritic processes of the melanocyte; (5) the transfer to and incorporation of melanosomes into the keratinocytes, either as single, discrete particles or as complex, aggregated particles; and (6) the degradation of these melanosomes within the keratinocytes [Jimbow et al, 1993].

Melanogenesis has been extensively reviewed by Prota [1988]. While tyrosinase is the major enzyme responsible for conversion of tyrosine to dopa (tyrosine-hydroxylation reaction) and the oxidation of dopa to dopaquinone (dopa-oxidase reaction), other tyrosinase-related cofactors are required. It is presumed that these cofactors play an important role in the regulation of melanogenesis, and are under the influence of several genetic or postregulatory factors [Bennett, 1993]. One cofactor is the tyrosinase-related protein-1 (TRP-1), a 75-kDa protein with structural homology of approximately 50% to tyrosinase. Its expression is directly correlated to the maturation of the melanosome, and needs to be expressed along with tyrosinase in order to form black melanin.

The molecular structure is similar to tyrosinase, but has been found to lack tyrosinase activity. Nonfunctional mutations of this protein may lead to either complete loss of melanization, or cell death (B^w, or white-based Brown mutation in mice), presumably through disruption of melanosomal membrane structure [Johnson and Jackson, 1992].

Gene sequencing performed while searching for flanking and controlling elements 5' to the tyrosinase and TRP-1 gene have identified several transcriptional regulatory motifs and palindromes, however, none were considered unique to these genes or melanocyte-specific [Bennett, 1993].

BIOLOGY OF MALIGNANT MELANOMA

Ontogeny of Melanocytic Nevi

The abnormal, pathological collection of epidermal melanocytes within the skin can be divided into two major groups: melanocytic nevus, and malignant melanoma. The following is a definition of these terms.

The initial proliferation of melanocytes within the epidermis is called an epidermal melanocytic nevus. A nevus, by definition, is a collection or proliferation of normal cells within a location where these cells are commonly found. Normally, these appear within one year of life, with new ones appearing until about 20 years of age. After a period of development, any given nevus will follow along one of two pathways. The first is the persistence of a flat 1 to 5 mm uniform brown nevus that does not change during life (lentiginous melanocytic nevus) [Clark et al, 1984]. The second pathway incorporates some progressive enlargement, with some elevation. Once the lesion reaches about 4 mm in diameter, lateral expansion ceases, and the lesion becomes globoid. Then over the next 50 - 60 years, the lesions soften and lose pigmentation, until they either involute or remain as a skin tag.

Commonly, the melanocytes in lentiginous melanocytic nevi are arranged either along the basal membrane, or in small nests at the tip of the rete ridges. Conversely, nevi undergoing progressive differentiation acquire these early stages of differentiation, only to develop more obvious compact, rounded nevus nests within the superficial dermis. At this stage, the nevus has cells within both the dermis and epidermis surrounding the basal membrane, and are called junctional, or compound nevi. As the nevus cells within the dermis progress deeper into the dermis, the epidermal proliferation is lost, until only the dermis contains the nevus cells, which is now called the dermal nevus. These cells deeper within the dermis take on a Schwann cell appearance, with elongation of the cells and the appearance of fascicles, and undergo what is called Schwannian differentiation. Furthermore, some cells take on an appearance similar to tactile nerve endings, and has been called neurotization. This signifies the terminal stage of nevus differentiation, and once complete, the overlying skin appears normal.

Rarely, some new nevi appear at mid-life. These lesions have an interesting life cycle, for studies on these nevi suggest that their appearance is due to a reversal of the differentiation pathway, back to the less differentiated junctional nevus state. Farber and Cameron [1980] suggested that these nevi may be analogous to the experimental lesions that may be "recalled". He observed that the hyperplastic nodules seen during liver carcinogenesis, which characteristically disappear via differentiation, may be recalled when the liver is again injured by the original inductive mechanism.

Ontogeny of Malignant Melanoma

Despite many differences, one feature common to all malignancies is the ability to invade surrounding tissue and form secondary metastatic tumours [Rusciano and Burger, 1992]. From a clinical point of view, most deaths from cancer are attributed to metastatic growth [Liotta et al, 1991]. Studies of metastasis reveal a complex cascade of sequential, interlinked steps [Rusciano and Burger, 1992; Garrido et al, 1993; Nicolson, 1993]. Cells that are malignantly transformed do not acquire all these abilities at the same time, but progress through sequential steps of transformation [Albino, 1992]. The identification of the cellular and molecular mechanisms involved in this transformation still remains the major challenge for cancer biologists [Rusciano and Burger, 1992].

The melanocytic nevi in some patients do not follow either of the earlier described pathways of normal development. If the nevus continues to grow radially along the basal membrane, beyond the 4 - 5 mm diameter, the pigmentation at the margin becomes irregular. The melanocyte growth pattern remians basilar, with minimal nesting, and the cells appear cytologically normal. This nevus pattern has been called a melanocytic nevus with persistent lentiginous melanocytic hyperplasia (aberrant differentiation).

Once the melanocytes demonstrate more cellular atypia, the nevus is now considered to be dysplastic. While substantial controversy remains surrounding the precise definition of this lesion, many investigators accept its existence. Three other histological features are described: 1) lamellar fibroplasia surrounding the nests of atypical cells; 2) concentric eosinophilic fibroplasia, giving a pink, ground-glass appearance to the surrounding stroma; and 3) patchy lymphocytic infiltrate around the blood vessels in the dermis. The presence of multiple dysplastic nevi in patients describes a syndrome called the familial dysplastic nevus syndrome, which carries an increased risk for subsequent development of melanoma of at least 26 to 400 times greater than the normal population, and is considered by many clinicians to be the harbinger of malignant melanoma precursors.

Up until this point, each of these nevi exhibiting aberrant differentiation may grow, compared to the normal differentiated nevi. However, this growth is not nonrestricted, that is, these aberrant nevi eventually cease growing, and then revert to a more normalized pattern. Thus, these nevi are not autonomous. In the next progressional step, from dysplastic nevus to radial growth melanoma, the restriction on growth is not present, but is partially, but indefinitely autonomous. However, if these lesions are not treated, evidence supports their inevitable progression to further stages of tumour progression. Thus, progression from melanocytic dysplasia to radial growth phase melanoma is an important step, as it is the first step of cancer, and progression is the rule, not the exception. Since the cells in the radial growth phase are only partially autonomous, these cells rarely are capable of metastatic growth. However, when the melanoma progresses from the radial growth phase to the vertical growth phase, these cells are now fully autonomous, and capable of sustaining a metastatic growth.

Pathophysiology of Malignant Melanoma and Melanocyte Differentiation

Malignant cells are thought to lose some phenotypic markers characteristic to the normal, differentiated cells [Liotta et al, 1991; Albino, 1992; Campbell, 1989; Tsukamoto et al, 1991]. Conceptually, the degree in differentiation of malignant cells is inversely related to the aggressiveness of their growth [Gray and Pierce, 1964]. During the transformation of melanocytic nevi to malignant melanoma, the aberrant/dysplastic nevus cells become larger with abundant eosinophilic cytoplasm, and appear more epithelioid than neuronal in phenotype. Furthermore, the ability to use tyrosinase to make melanin and melanosomes becomes progressively deteriorated and many of malignant melanocytes are finally incapable of making melanized melanosomes.

The loss of these phenotypic markers in melanocyte differentiation supports the idea that malignant transformation causes the progressive loss of the differentiation phenotype. In other words, cells which are progressively transformed may lose phenotypic markers that are expressed on normal cells. How this process occurs, and how the loss of differentiation is related to malignancy is not well understood.

Heterogeneity of Malignant Melanocyte Differentiation

Progression from melanocytic dysplasia to radial growth phase of malignant melanoma does not occur simultaneously. Rather, the alterations are focal and qualitative. Clinically, the first indication of melanoma progression is the appearance of small, discrete areas of increased pigmentation within the tan-brown coloured nevus nests. Histologically, these areas demonstrate small, focal collections of malignant cells within a large population of otherwise normal looking melanocytes. Thus, these changes are consistent with a mutational event occuring within the melanocytic dysplasia. Since, these areas are not synchronous, the development of multiple clones of dysplastic/malignant cells may occur within a single lesion. This multiple clonality can be the reason for the substantial heterogeneity in the colour observed in the established tumour.

Malignant melanoma is one of the representative cancers that have been carefully studied with respect to the heterogenous tumour progression and differentiation, particularly in the degree of pigmentation, cell morphology, antigenic profiles, and growth rate. The early and poorly differrentiated melanoma cells are nonpigmented, epithelioid, and express certtain surface markers. Late and highly differentiated melanoma cells are pigmented, dendritic, and express certain pigmentation-related markers. Importantly, phenotypic heterogeneity has been demonstrated among primary and metastatic lesions in the same patient, and among individual metastases from the same primary tumour [reviewed in Herlyn, 1990].

Tumour heterogeneity *in situ* extends to the expression of integrin cell adhesion molecules. Tissue sections of primary and metastatic melanomas show evidence of heterogeneity in the level of expression for at least some integrin α and ß subunits [Mortarini and Anichini, 1993]. Particularly, there is marked heterogeneity for some of the α subunits of ß1 and ß3 integrins [Mortarini and Anichini, 1993]. In one recent study, integrin subunit expression by cryostat sections of human melanoma tumours was examined using immunoperoxidase staining methods. The heterogeneity was predominantly found with α2- and α3-integrin subunits for all tumour stages, while α1- and α6-integrin subunit expression was confined mainly to metastatic lesions [Natali et al, 1991]. Five tumour clones which expressed multiple VLA molecules had the lowest melanin content, and expressed an epithelioid morphology [Anichini et al, 1990]. The data would suggest that cells in an early stage of differentiation may be characterized by an increased expression of multiple α-integrin subunits [Mortarini and Anichini, 1993]. Therefore, changes in integrin expression within tumours exhibiting heterogenous cell populations may belie a potentially significant role for integrin function in the biological process of malignant progression.

CELL-SUBSTRATE ADHESION AND MALIGNANT TUMOUR PROGRESSION

Association between Cell-Substrate Adhesion and Tumour Progression

Foulds [1969] established that the development of malignant tumours follows a stepwise alteration in cell behaviour and morphology. This profile of sequential phenotypic change represents the process of tumour progression. It is likely that a successful phenotype include a suitable array of integrin receptors that would directly determine the adhesive properties of cells and thus influence invasion and metastatic potential [Kramer et al, 1991a]. The ability of tumour cells to invade through the dermal-epidermal basement membrane and then penetrate into the interstitial extracellular matrix is dependent on their capacity to adhere to, interact with, and migrate through these matrices [Kramer et al, 1991a]. Selection pressures during the metastatic process would favour those cells that have enhanced capacity to attach to and migrate through cellular and extracellular matrix barriers [Kramer et al, 1991a].

The process of malignant transformation of normal cells has been shown to trigger alterations in integrin expression [Ruoslahti and Giancotti, 1989]. Little information is available about differences between normal melanocytes and malignant melanoma cells in their expression of adhesion receptors [Kramer et al, 1991a]. It has been proposed that the degree of differentiation of melanoma cells influences their capacity to invade and metastasize. Given the known heterogeneity of tumour cell populations, it might be expected that there is also heterogeneity in integrin profiles. In fact, one study has examined the integrin profiles of a number of individual clones isolated from a human melanoma [Anichini et al, 1990]. These clones showed extreme heterogeneity in the expression of the β1 integrins. Futhermore, clones of cells that were poorly differentiated tended to possess more α-integrin subunit diversity, whereas clones of more differentiated cells generally had fewer types of α-integrin subunits, and α3-integrin was the predominant integrin [Kramer et al, 1991a].

It may be reasonable, then, to hypothesize that non-metastatic melanoma would express multiple receptors that could bind firmly to basement-membrane-specific components, such as laminin and type IV collagen. Yet, interstitial invading tumour cells at the primary tumour site must be highly motile in order to reach neighbouring vascular beds for subsequent dissemination. Thus, the cells must also express receptors that can efficiently interact with interstitial matrix components, such as collagen Types I and III and fibronectin [Kramer et al, 1991a]. Locomotory cells form weak and transient close contacts and the integrins remain as a diffuse distribution [Duband et al, 1988; Akiyama et al, 1989].

The diverse number of potential integrin adhesion receptors and their promiscuous ligand specificity suggests that there may not necessarily be one specific integrin profile that favours metastasis [Kramer et al, 1991a]. Instead, the combination of several integrin subunits expressed in union, or sequentially, may provide a suitable signal which would direct the cell behaviour. Correspondingly, any alteration in the signalling capacity of these integrins may indicate the beginning of a new phase of tumour progression. At present, there are some intriguing prospects regarding the role of modulation of intercellular signalling via integrins, however, the evidence is far too preliminary to provide a complete picture.

Biology of Integrins as Cell-Substrate Adhesion Molecules: The universal concepts of integrins

There is one particular class of adhesion molecules which has captured the attention of investigators for almost 14 years, namely the integrins. In 1986, Hynes and his coworkers proposed the unifying term 'integrin' to denote the role of these adhesive receptors as "... an integral membrane complex involved in the transmembrane association between the extracellular matrix and the cytoskeleton" [Tamkun et al, 1986]. Integrins have been implicated in regulating cell adhesion, and in controlling cell behaviour on specific substrates. Since integrins are capable of multiple roles, integrins have been intensively studied [Hynes, 1992].

The biological importance of integrins is underscored by the high degree of conservation of homologous domains across genera. Cells from the fruit fly, *Drosophila*, express adhesion molecules which have amino acid sequences containing up to 75% homology with mamalian integrins. The sequences and genes of *Drosophila* integrins are about as closely related as those of the most divergent vertebrate subunits. It is thus clear that integrins have arisen at a very early point in evolution, before divergence of the protostome and deuterostome lineages [Hynes, 1992].

All integrins expressed on the cell surface are heterodimers [Hynes, 1992]. Conclusive evidence exists which demonstrates that individual integrin subunits are not translocated or expressed onto the cell surface [Gailit and Clark, 1993]. Although 8 ß-integrin subunits and 14 α-integrin subunits could, in theory, associate to give more than 100 integrin heterodimers, the actual diversity appears to be much more restricted [Hynes, 1992]. Assembly of integrins is a selective process, since not all possible combinations are seen within a cell. One explanation for this process is that dimerization is governed by the relative affinities of competing ß-integrin subunits for the available α-integrin subunit, and by the relative amounts of competing ß-integrin subunits [Gailit and Clark, 1993].

Once the α- and ß-integrin subunits are combined, the integrin heterodimer is processed in the Golgi complex and converted to the mature, fully glycosylated receptor before being transported to the cell surface [Gailit and Clark, 1993]. However, the oligosaccharide processing does not seem to be required for assembly of the integrin heterodimer, or for transport to the cell surface and insertion into the cell membrane [Akiyama et al, 1990; Cheresh, 1991]. Interactions between the α- and ß-integrin subunits do not rely on the transmembrane or cytoplasmic domains; this conclusion is also supported by the selective α-integrin subunit associations with chimeric ß-integrin subunits [Hynes, 1992]. Therefore, the current picture is that the N-terminal domains of α and ß subunits combine to form a ligand-binding head on each integrin [Hynes, 1992].

Both integrin subunits are required for binding to ligands [Gailit and Clark, 1993]. Characteristic of all ß-integrin subunits is a four-fold repeat of a cysteine-rich segment believed to be internally disulfide-bonded [Hynes, 1992]. Most importantly, divalent metal cations are required for ligand binding. The α-integrin subunits all contain a seven-fold repeat of a homologous segment; the last three of four of these repeats contain sequences that likely contribute to the divalent metal cation-binding properties of these subunits. Divalent metal cations are essential for integrin receptor function [Hynes, 1992]. The biochemical and biophysical properties of the divalent metal cations can affect both the affinity and the specificity for the ligand, and divalent cations are necessary for αß subunit association by some integrins [Hynes, 1992]. Finally, the binding of an integrin heterodimer to its ligand can be affected by many auxiliary factors, including the glycosylation state of the integrin subunits [Akiyama et al 1990], and the association of the integrin heterodimer with lipids and gangliosides in the cell membrane [Cheresh, 1991; Bennett, 1993].

Integrins are not permanently expressed on the cell surface, but instead, undergo either selective degradation, or recycling. A large portion of the ß1-integrin precursor pool, perhaps as much as 60 to 70%, is degraded without ever being expressed [De Strooper et al, 1991; Roberts et al, 1988]. Proteolysis is a second possibility for removal of an integrin from the cell surface [Gailit and Clark, 1993]. Consistent with a model of compact folded domains, integrins are fairly resistant to proteolysis while expressed on intact cells [Hynes, 1992]. A second possibility is the endocytosis of integrin subunits. A study of cell-cell contact sites in a variety of cell types showed that α3ß1 integrin was internalized via coated pits and concentrated later in large vesicles [Kaufmann et al, 1989]. Another study concluded that hemidesmosomes, containing α6ß4, are endocytosed by basal keratinocytes after trypsin treatment which separates the epidermal cell layer from the dermis [Takahasi et al, 1985]. Thus, cells can restrict their adhesion to surfaces by regulating the expression of integrin subunits, or by removing integrin subunits already existing on the cell surface. The mechanisms which control such activities are not known.

Biochemical studies on integrin function using *in vitro* culture cells have demonstrated that the receptor/ligand system is characterized by redundancy in the number of receptors for the same extracellular matrix ligand [Table I-1] [Zambruno et al, 1993]. However, the existence of multiple receptors for the same extracellular matrix ligand does not mean that each receptor interacts in the same way with that ligand [Mortarini et al, 1991].

The expression of multiple integrin receptors for the same extracellular matrix ligand by the same cell type is a mechanism which allows for the cooperation by integrins to mediate the same function [Mortarini et al, 1991]. Integrins can deliver to the cytoplasmic side of the cell membrane a variety of signals originating from the surrounding matrix, thus activating a chain of intracellular events leading to a measurable biological response [Mortarini et al, 1991]. As a consequence of these activation signals delivered to the cell, the integrins can dynamically change their avidity for the specific ligand, thus behaving as flexible modulators of cellular adhesion. Integrins can, therefore, exist in either active or inactive form that determines the avidity for the specific ligands [Hynes, 1992].

The ligand avidity of an integrin also can depend upon the activation state of the cell [Gailit and Clark, 1993]. In some cell types, the ß1-integrins are constitutively activated [Gailit and Clark, 1993]. However, it is not known how cells are capable of modulating the activation state of the integrin subunits. Nonetheless, changes in conformational state of the subunits as a result of ligand binding, or by occupation of the cation-binding site could be implicated.

Integrin Expression in Normal Epidermal Development

Integrins appear to play a role in the normal development of the human epidermis. Studies have been conducted on fetal epidermis to map the patterns of integrin expression at various stages of embryonic development. In fetal epidermis, ß1-integrin is uniformly distributed around the cell membrane [Ryyanen et al, 1991; Hertle et al, 1991]. In contrast, ß1-integrin expression in the adult epidermis seems to be concentrated at the lateral and apical surfaces of the cell and is rarely expressed on the basal surface [Ryyanen et al, 1991; Larjava et al, 1990]. The $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$-integrin subunits form functional complexes only with the ß1-integrin subunit [Gailit and Clark, 1993].

The $\alpha1$-integrin subunit is strongly expressed at early stages of embryonic development, but its expression diminishes after about 14 weeks of gestation [Hertle et al, 1991]. In the postnatal epidermis, α1-integrin has not been identified by conventional immunohistochemistry [Carter et al, 1990; Konter et al, 1989; Klein et al, 1990], or by immunoelectron microscopy [Zambruno et al, 1991]. The α3-integrin subunit was detected throughout development [Hertle et al, 1991]. Although early expression of α2-integrin was weak or patchy, after stratification its expression was very similar to that of α3-integrin [Hertle et al, 1991; Zambruno et al, 1991]. In the neonate and adult epidermis, α2-, α3-, and β1-integrin have all been found in suprabasal cells [Larjava et al, 1990; Konter et al, 1989; Klein et al, 1990; DeLuca et al, 1990], although their detection may depend upon the particular monoclonal antibody employed [Carter et al, 1990].

Expression of the α5-integrin subunit begins relatively late during epidermal development in the embryo [Hertle, et al, 1991; Wayner et al, 1988; Peltonen et al, 1989]. The αv-integrin subunit is first expressed in fetal skin with the onset of stratification [Hertle et al, 1991]. The existence of αvβ5 in the skin has not been directly proven, and, in fact, no attempt to identify β5 in skin has been reported [Gailit and Clark, 1993]. The hemidesmosome-related α6- and β4-integrin subunits are both strongly expressed throughout gestation [Ryyanen et al, 1991; Hertle et al, 1991; Zambruno et al, 1991]. The β4-integrin subunit seems to have a very limited tissue distribution. It has been found only in epithelial tissues along the basement membrane, and in Schwann cells of peripheral nerves [Sonnenberg et al, 1990]. Therefore, integrin subunit expression is tightly controlled during embryonic epidermal development, and the selective appearance and disappearance of specific subunits implies that the functional role of these subunits is unique to the stage of embryonic development.

Integrin expression is also implicated in regulating cell differentiation of the normal epidermis. For example, the expression of α5β1 *in situ* is low and the integrin is not functional in the epidermis. However, α5β1 expression by epidermal cells can be reversibly induced in culture [Gailit and Clark, 1993]. Furthermore, terminal differentiation of basal keratinocytes apparently occurs in two stages. The ligand binding function of the β1-integrins is reduced in the first stage, while the cell surface expression of the integrins is reduced later in the second stage [Gailit and Clark, 1993]. During the process of wound healing, α5β1 integrin expression is greatly increased on migrating keratinocytes, but then decreases after the wound surface is covered by basal keratinocytes [Gailit and Clark, 1993]. Thus, increased α5β1 expression on wound healing may prevent unwanted terminal differentiation [Gailit and Clark, 1993].

Integrin Expression in Tumour Progression *in vivo*

The expression of integrin subunits on tumours derived *in vivo* have been studied, in order to determine whether certain subunits regulated tumour behaviour. The hypothesis was that tumour cells would alter their adhesion to surrounding cells and stroma, thereby allowing the tumour cells to detach from their surrounding and invade. And while the literature is somewhat variable, several investigators have shown that certain integrin subunits change their expression during tumour progression.

Malignant epithelial cells which undergo metastasis must first decrease their attachment to their neighbouring cells before they can invade the underlying stroma [Gailit and Clark, 1993]. Since the integrins $\alpha2\beta1$ and $\alpha3\beta1$ are highly expressed by basal epithelial cells, and are implicated in cell-cell interaction, changes in the expression of these integrins would be of particular interest. Expression of $\alpha2\beta1$ and $\alpha3\beta1$ in squamous cell carcinoma, a metastatic form of epithelial cancer, shows a loss of the polarized phenotype, and a decrease in overall expression of these integrins on tumour cells which invade the stroma [Kaur and Carter, 1992]. Conversely, the integrin expression by basal cell carcinoma, a malignancy which rarely metastasizes, is either normal [Peltonen et al, 1989], or is increased [Stamp and Pignatelli, 1991]. Thus, the functional loss of cell-cell interaction through $\alpha2\beta1$ and $\alpha3\beta1$ may be one of the primary changes during tumour progression.

Patterns of expression by other ß-integrin subunits have revealed that the ß3-integrin family may be expressed only during certain stages of tumour progression. Initial studies by McGregor and colleagues [1989] reported that ß3-integrin was detectable in most sections of malignant melanoma tumours, but was undetectable in normal melanocytes, melanocytic nevi, and dysplastic nevi. This finding was later confirmed by the extensive study performed by Albelda and colleagues [Kramer et al, 1991a], who examined tumour cells at all stages of tumour progression. They found that ß3-integrin expression was low in normal melanocytic nevi and melanomas in the radial growth phase, but increased significantly in the vertical growth phase and metastatic growth phases. Since the distinction between melanomas of early VGP and RGP is difficult, the expression of the ß3-integrin by melanoma cells which could potentially metastasize could be useful as an indicator for cells entering this phase of malignant progession [Albelda et al, 1990; McGregor et al, 1989].

Integrin Expression in Tumour Cells *in vitro*

Integrin expression has been studied in normal and metastatic malignant melanocytes cultivated *in* *vitro.* Immunohistochemical and immunoprecipitation studies found that normal melanocytes express ß1-integrin subunit, predominantly in association with the α3-integrin subunit [Albelda et al, 1990]. These cells also express, albeit with some heterogeneity, the α1, α2, and α5-integrin subunits [Albelda et al, 1990]. The αv-integrin subunit was also expressed on cultured melanocytes, in association with ß3-integrin [Zambruno et al, 1993], although it could also be associated with the ß5-integrin subunit [Kramer et al, 1991a]. Furthermore, these studies failed to detect α4, α7, ß2, or ß6-integrin [Albelda et al, 1990; Zambruno et al, 1993; Felding-Habermann et al, 1992]. Thus, normal melanocytes express a limited repertoire of integrin subunits.

As each integrin dimer has an affinity for particular substrates, normal melanocytes express several integrins which can identify the same substrate. For example, α2ß1 recognizes collagen and laminin, whereas α3ß1 recognizes ligands on fibronectin, collagen, laminin, and an epidermal substrate called epiligrin [Hynes, 1992]. This expression of multiple receptors for the same ligand on the same cell type would provide a cooperative signalling mechanism that would allow integrins to modulate cell adhesion to substrates [Mortarini and Anichini, 1993].

The comparison in integrin expression between normal melanocytes and metastatic malignant melanoma cells has yielded a wide variety of results. The most consistent change reported is the increased expression of α4-integrin [Mortarini and Anichini, 1993; Albelda et al, 1990] and ß3-integrin [Albelda et al, 1990; Zambruno et al, 1993] by metastatic melanoma cells, compared to normal melanocytes. Specifically, variants of the same human melanoma expressing different metastatic potential in nude mice indicated a marked increase in α4-integrin expression [Mortarini and Anichini, 1993]. The ligand for α4-integrin is the CS-1 domain of fibronectin, and the vascular cell adhesion molecule-1 (VCAM-1), which is expressed on activated endothelial cells. Thus, melanoma cells may acquire the ability to enter the vascular compartment of the tissue, and progress to the metastatic phase of tumour progression through the expression of α4-integrin.

Expression of ß3-integrin is also correlated with metastatic ability. Melanoma cells in the vertical and metastatic growth phases expressed greater amounts of ß3-integrin than normal melanocytes or melanoma cells in the radial growth phase [Albelda et al, 1990; McGregor et al, 1989]. A functional role of the αvß3 receptor might be implicated in this process; melanoma variants lacking the ß3-integrin are much less tumourigenic in nude mice than ß3-integrin positive cells [Felding-Habermann et al, 1992]. Therefore, the examination of the function of ß3-integrin would be of importance in tumour progression.

Kramer and colleagues [1991a] described the presence of a novel α-integrin subunit which is expressed solely by malignant melanoma cells, but not by normal melanocytes. This integrin, called $\alpha 7$, is expressed by metastatic malignant melanocytes [Albelda et al, 1990; Kramer et al, 1991b]. It is not expressed in normal melanocytes [Albelda et al, 1990; Zambruno et al 1993; Kramer et al, 1991b]. While the function of $\alpha 7\beta 1$ is not fully understood, its presence during tumour progression implies an important role.

Other integrin subunits have also revealed changes in their expression during tumour progression, although the evidence is less conclusive. Increased expression of $\alpha 1$ [Mortarini et al, 1991], $\alpha 2$ [Albelda et al, 1990; Mortarini et al, 1991; van Muijaen et al, 1991], and $\alpha 6$ [Mortarini et al, 1991] has been reported in metastatic melanoma cell lines. In many of these studies, a relationship between the integrity of the basement membranes and expression of integrins appeared to exist; those tumours that were more invasive and lacked intact basement membrane tended not to express basement-membrane protein binding integrins, such as $\alpha 6$ [Albelda, 1993].

Melanoma cells can acquire the expression of integrin subunits such as $\alpha 4$ and $\alpha 7$ which are not normally found on normal melanocytes. Additionally, melanoma cells can increase the levels of expression of other subunits that are also found on normal melanocytes, such as $\alpha 1$, $\alpha 2$, $\alpha 5$, and αv-integrin [Albelda et al, 1990]. However, studies of other tumour types have not demonstrated the ability to acquire new integrins. Kaur and Carter [1992], in their study of virally-transformed squamous cells, found that all the integrin subunits expressed by normal keratinocytes were found on the transformed keratinocytes. Their conclusion was that transformation of epidermal cells did not result in the functional alteration of integrins [Kaur and Carter, 1992].

In summary, the pattern of integrin expression on tumour cells *in vitro*, like that *in situ*, appears to be complex and dependent on the tumour type [Albelda et al, 1990]. Previous data indicate that the association of integrin expression with tumour progression of malignant melanoma does not involve a single receptor, and that an increased expression is not necessary. Rather, it appears that the whole repertoire of integrin receptors is subjected to a complex shift as the neoplastic population acquires an invasive and metastatic capacity [Mortarini and Anichini, 1993]. Changes in cell adhesion observed during tumour progression may have more to do with the functional activity of the integrins expressed. Functional modulation of integrins without changes in surface expression is an area which is under closer investigation. A study of cultured human cells demonstrated, using immunoelectron microscopy, that $\alpha 3\beta 1$ was strongly associated with intercellular contact sites [Kaufmann et al, 1989].

Functional Studies of Integrin-Mediated Cell-Subtrate Adhesion in Tumour Cells in vitro

Integrins appear to be the major receptors by which cells adhere to extracellular matrices, and some integrins also mediate important cell-cell adhesion events [Gailit and Clark, 1993]. Indeed, experiments using melanoma cell lines have demonstrated several integrin subunits are responsible for adhesion to substrates. However, there has been substantial heterogeneity in the results of these experiments. Some of the apparently conflicting results that demonstrate both enhanced and diminished tumour cell adhesion during tumour progression may be attributed to the experimental systems used [Albelda, 1993]. Thus, any comparison of cell-substrate adhesion between cell lines should be interpreted with caution, and the experimental parameters by which the assays were performed should be carefully scrutinized.

Cell-substrate adhesion can be modulated by one of two mechanisms: (1) the physical location of the adhesion molecules within the membrane-cytoskeletal organization, and (2) the communication between different adhesion molecules. The first method involves some specific substructure. For example, cells which organize their integrin receptors into specialized adhesion-cytoskeletal structures called adhesion plaques or stress lines, are capable of withstanding greater detachment forces, than cell lines which are incapable of forming such structures. Normal melanocytes and malignant melanoma cell lines do not form any specialized adhesion structures, such as adhesion plaques.

The second method for modulation of adhesion is by communication between other adhesion molecules. A putative candidate integrin is the $\alpha v\beta 3$ molecule. For example, monoclonal antibodies against $\alpha v\beta 3$ integrins did not interfere with melanocyte adhesion to fibronectin or vitronectin, but potently inhibited cell spreading. Thus, integrins which are expressed on cells may not have a direct role in cell adhesion, but may be responsible for modifying cell adhesion or other behaviours. The mechanisms by which this occurs is not understood.

Finally, the major obstacle in making any sweeping generalizations about cell adhesion molecules and tumour progression is that the process of successful metastasis is inherently complex, with increased adhesion at one phase, and decreased adhesion at another phase [Albelda, 1993]. Experiments using cell lines expressing several integrin receptors for a single extracellular component have shown that only one integrin receptor was mediating most of the binding capacity [Mortarini et al, 1991b]. However, this same integrin receptor may not adhere to the same extracellular component in different cell lines [Mortarini et al, 1991a]. This suggests that neoplastic transformation can not only change the integrin profile of melanocytes, but also may shift the position in the hierarchy of roles ascribed to the different integrins [Mortarini et al, 1991a].

SUMMARY

Human epidermal melanocytes are of neural crest derived cells which undergo a highly regulated process of embryonic migration and cellular differentiation, which is epitomized by the formation of a symbiotic unit with the neighbouring cells, and the production of a secretory organelle, called the melanosome. The melanocytes undergo a well characterized differentiation pattern throughout the life of the host. Occasionally, this differentiation pattern is aborted or transformed, which is seen during neoplastic transformation to malignant melanoma. This transformation can be assessed according to mitotic activity, and by regression of the differentiated phenotype to a more primitive, embryonic phenotype.

The basal positioning of melanocytes on the basement membrane within the EMU is critical for its proper functioning. Proper expression and function of adhesion molecules would be responsible for the early embryonic formation of the EMU. Furthermore, alterations of melanocyte positioning within the epidermis, presumably through altered adhesion regulation, is one of the first stages associated with malignant transformation. Therefore, there has been substantial interest in the role of integrin expression and function during tumour progression. However, studies have been hampered by the heterogeneity of tumours. Also, studies comparing normal melanocytes with melanoma cell lines have been hampered by heterogeneity of metastatic potential and tumour progression, making interpolation very suspect. Only recently have melanoma cell lines been established from each major phase of tumour progression. However, the expression and function of integrins on these cell lines has not been characterized.

The role of the loss of the cellular differentiation phenotype with tumour progression has always been implied, but never conclusively demonstrated with malignant melanoma. The characterization of the differentiation phenotype of cell lines derived from different phases of tumour progression has not been reported. Furthermore, cell lines can show some variability in their differentiation following exposure to reversible differentiation-induction agents, such as 5-bromo-2-deoxyuridine, or other cytokines, like the α-melanocyte stimulating hormone. It is not known whether these melanoma cell lines are sensitive to these agents, and to what degree these agents would alter the differentiation phenotype.

Finally, if integrins are indeed responsible for some of the changes observed during tumour progression *in situ*, it may be possible that the integrin expression and function would be linked to cellular differentiation. It may also be reasonable to expect that integrin function would change according to the phases of tumour progression, and reflect the ability to recognize and adhere to extracellular matrices that are common to the cellular environment. If this is true, then the determination of the dominant integrin profile by a tumour may predict the clinical prognosis, and therapies directed at limiting the integrin function at later stages of metastasis may be useful in preventing successful metastasis within the host.

Table I-1: Integrin Subunit Family Examined in this Study

Subunits[a]	Ligands[b]	Binding Site[c]
α2	Collagens, Laminin	DGEA
α3	Fibronectin, Collagens, Laminin	RGD
α4	Fibronectin, VCAM-1	EILDV
α5	Fibronectin	RGD
αv	Vitronectin, Fibronectin, Collagen, Fibrinogen, Thrombospondin, Osteopontin, von Willebrand factor	RGD
ß1	Vitronectin, Fibronectin, Collagen, Fibrinogen, Thrombospondin, Osteopontin, von Willebrand factor, Laminin	Multiple sites
ß2	ICAM-1, ICAM-2, Fibrinogen, C3b component of Complement	Multiple sites
ß3	Vitronectin, Fibronectin, Collagen, Fibrinogen, Thrombospondin, Osteopontin, von Willebrand factor	RGD
ß4	Laminin	Unknown

a = Integrin subunits identified by murine monoclonal antibodies in this study
b = Ligand recognition may be cell-specific
c = Amino acid sequence on extracellular matrix that is recognized by the integrin subunit
DGEA = Defined in Collagen Type I only
RGD = Arginine-Glycine-Aspartate
EILDV = Defined on in the alternatively spliced V segment of Fibronectin
ICAM = Intercellular Adhesion Molecule

compiled from [Hynes, 1992; Albelda, 1993]

BIBLIOGRAPHY

Akiyama SK, SS Yamada, WT Chen, KM Yamada. Analysis of fibronectin receptor function with nomoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. J.Cell Biol. 109:863-875, 1989.

Akiyama SK, K Nagata, KM Yamada. Cell surface receptors for extracellular matrix components. Biochim. Biophys Acta 1031:91-110, 1990.

Albelda SM, SA Mette, DE Elder, R Stewart, L Damjanovich, M Herlyn, CA Buck. Integrin distribution in malignant melanoma: association of the ß3 subunit with tumor progression. Cancer Res. 50:6757-6764, 1990.

Albelda S. Role of integrins and other cell adhesion molecules in tumor progression and metastasis. Lab.Invest. 4-17, 1993.

Albino AP. The role of oncogenes and growth facators in progressive melanoma-genesis. Pigment Cell Res. Suppl 2:199-218, 1992.

Anichini A, M Mortarini, E Berti, G Parmiani. Multple VLA antigens on a subset on melanoma clones. Human Immunol. 28:119-122, 1990.

Bennett DC. Genetics, Development, and Malignancy of Melanocytes. Int. Rev. Cytology 146:191-260, 1993.

Bennett JS. Integrin structure and function in hemostasis and thrombosis. Ann. NY Acad. Sci. 614:214-228, 1991.

Birbeck MSC, Barnicot NA. Electron microscope studies on pigment formation in human hair follicles, in M Gordon (ed): Pigment Cell Biology New York, Academic Press, 1959, p 549-561.

Campbell S. Melanogenesis of avian neural crest cells *in vitro* is influenced by external cues in the periorbital mesenchyme. Development 106:717-726, 1989.

Carter WG, EA Wayner, TS Bouchard, P Kaur. The role of integrins α2ß1 and α3ß1 in cell-cell and cell-substrate adhesion of human epidermal cells. J. Cell Biol 110:1387-1404, 1990.

Cheresh DA. Structure, function and biological properties of integin $\alpha v\beta 3$ on human melanoma cells. Cancer Metast. Rev. 10:3-10, 1991.

Clark WH, DE Elder, D Guerry, MN Epstein, MH Greene, M van Horn. A study of tumor progression: The precursor lesions of superficial spreading and nodular melanoma. Human Pathol. 15:1147-1165, 1984.

DeLuca M, RN Tamura, S Kajiji, S Bondanza, P Rossino, R Cancedda, PC Marchisio, V Quaranta. Polarized integrin mediates human keratinocyte adhesion to basal lamina. Proc.Natl.Acad.Sci.(USA) 87:6888-6892, 1990.

De Strooper B, FV Leuven, G Carmelier, H van den Berghe, JJ Cassiman. Cultured human fibroblasts contain a large pool of precursor $\beta 1$-integrin but lack an intracellular pool of mature subunit. Eur. J. Biochem. 199:25-33, 1991.

Duband JL, GH Nuckolls, A Ishihara, T Hasegawa, KM Yamada, JP Thiery, K Jacobson. Fibronectin receptor exhibiting high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in stationary cells. J.Cell Biol. 107:1385-1396, 1988.

Farber E, R Cameron. The sequential analysis of cancer development. In Klein G, S Weinhouse (eds): Advances in Cancer Research, vol 31. New York, Academic Press, 1980, pp 125-226.

Felding-Habermann B, BM Mueller, CA Romerdahl, D Cheresh. Involvement of integrin αv gene expression in human melanoma tumorigenicity. J.Clin.Invest. 89:2018-2022, 1992.

Fitzpatrick TB, AS Breathnach. Das epidermale Melanin-Einheit-System. Dermatol. Wochenschr. 147:481-489, 1963.

Foulds L. Neoplastic development Vol. 1, Academic Press, New York, 1969, pp 69-93.

Frenk E, JP Shellhorn. Zur Morphologie der epidermalen Melanineinheit. Dermatologica 139:271-277, 1969.

Gailit JO, RAF Clark. Integrins. Adv. Dermat. 8:129-153, 1993.

Garrido F, T Cabrera, A Concha, S Glew, F. Ruiz-Cabello, PL Stern. Natural history of HLA expression during tumor development. Immunol. Today 14:491-499, 1993.

Gray JM, GB Pierce. Relationship between growth rate and differentiation of melanoma *in vivo.* J.Natl.Cancer Instit. 32:1201-1210, 1964.

Herlyn M. Human melanoma: development and progression Cancer Metas. Rev. 9:101-112, 1990.

Hertle MD, JC Adams, FM Watt. Integrin expression during human epidermal development *in vivo* and *in vitro* Development 112:193-206, 1991.

Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11-25, 1992.

Jimbow K, Kukita A. Fine structure of pigment granules, in Kawamura S (ed): Biology of Normal and Abnormal Melanocytes Tokyo, Univ. of Tokyo Press, 1971, p 171.

Jimbow K, M Takahashi, S Sato. Ultrastructural and cytochemical studies of melanogenesis in melanocytes of normal hair matrix. J.Electron Microsc.(Tokyo) 20:87-92, 1971.

Jimbow K, SI Roth, TB Fitzpatrick. Mitotic activity in non-neoplastic melanocytes *in vivo* as determined by histochemical, autoradiographic and electron microscopic studies. J. Cell Biol. 66:663-670, 1975.

Jimbow K, WC Quevado, TB Fitzpatrick. Some aspects of melanin biology: 1950-1975. J. Invest.Dermat. 67:72-89, 1976.

Jimbow K, WC Quevado, TB Fitzpatrick, G Szabo. Biology of Melanocytes. in Fitzpatrick TB, AZ Eisen, K Wolff, IM Freedberg, KF Austen (eds): Dermatology in General Medicine. 4th ed. 1993. pp. 261-289.

Johnson R, J Jackson. Light is a dominant mouse mutatuion resulting in premature cell death. Nature (Genet.) 1:226-229, 1992.

Kaufmann R, D Frosch, C Westphal L Weber, CE Klein. Integrin VLA-3: ultrastructural localization at cell-cell contact sites of human cell cultures. J. Cell Biol. 109:1807-1815, 1989.

Kaur P, WG Carter. Integrin expression and differentiation in transformed human epidermal cells is regulated by fibroblasts. J. Cell Sci. 103:755-763, 1992.

Klein CE, T Steinmayer, JM Mattes, R Kaufmann, L Weber. Integrin of normal human epidermis: differential expression, synthesis and molecular structure. Br.J. Dermat. 123:171-178, 1990.

Konter U, I Kellner, E Klein, et al. Adhesion molecule mapping in normal human skin. Arch Dermat. Res. 281:454-462, 1989.

Kramer RH, M Vu, Y-F Cheng, DM Ramos. Integrin expression in malignant melanoma. Cancer Metast. Rev. 10:49-59, 1991a.

Kramer RH, MP Vu, Y-F Cheng, DM Ramos, R Timpl, N Waleh. Laminin-binding integrin $\alpha 7\beta 1$: Functional characterization and expression in normal and malignant melnaocytes. Cell Regul. 2:805-817, 1991b.

Larjava H, J Peltonen, SK Akiyama, SS Yamada, HR Gralnick, J Uitto, KM Yamada. Novel function for ß1 integrins in keratinocyte cell-cell interactions. J. Cell Biol. 110:803-815, 1990.

Lerner AB. Neural control of pigment cells, in T Kawamura (ed.): Biology of Normal and Abnormal Melanocytes. Tokyo, Univ of Tokyo Press, 1971, p 3.

Liotta LA, WG Slater-Stevenson, PS Steeg. Cancer invasion and metastasis: positive and negative regulatory elements. Cancer Invest. 9:543-551, 1991.

Lynch SA, BN Bouchard, S Vijayasanadhi, H Yuasa, AN Houghton. Antigens of melanocytes and melanoma. Cancer Metastasis Rev. 10:141-50, 1991.

McGregor BC, JL McGregor, LM Weiss, GS Wood, CH Hu, H Boukerch, RA Warnke. Presence of cytoadhesins (IIb-IIIa-like glycoproteins) on human metastatic melanomas, but not on benign melanocytes. Am.J.Clin.Pathol. 92:495-499, 1989.

Markert CL Silvers WK. The effects of genotype and cell environment on melanoblast differentiation in the house mouse. Genetics 41:429-439, 1956.

Maul GG Golgi-melanosome relationship in human melanoma *in vitro*. J Ultrastruct. Res. 26:163-176, 1969.

Maul GG, JA Brumbaugh. On the possible function of coated vesicles in melanogenesis of the regenerating fowl feather. J. Cell Biol. 48:41-48, 1971.

Mortarini R, A Anichini, G Parmiani. Heterogeneity for integrin expression and cytokine-mediated VLA modulation can influence the adhesion of human melanoma cells to extracellular matrix proteins. Int. J. Cancer 47:551-559, 1991a.

Mortarini R, A Anichini, G Parmiani. Heterogeneity for integrin expression and cytokine-mediated VLA modulation can influence the adhesion of human melanoma cells to extracellular matrix proteins. Int.J. Cancer 47:551-559, 1991b.

Mortarini R, A Anichini. From adhesion to signalling: roles of integrins in the biology of human melanoma. Melanoma Res. 3:87-97, 1993.

Natali PG, MR Nicotra, R Cavaliere, D Giannarelli, A Bigotti. Tumour progression in human malignant melanoma is associated with changes in $\alpha6\beta1$ laminin receptor. Int. J. Cancer 49:168-172, 1991.

Nicolson GL. Cancer progression and growth: relationship of paracrine and autocrine growth mechanisms to organ preference of metastasis. Exp. Cell Res. 204:171-180, 1993.

Novikoff AB, A Albala, L Biempica. Ultrastructure and cytochemical observations on B-16 and Harding-Passey mouse melanomas - the origin of premelanosomes and compound melanosomes. J. Histochem. Cytochem. 16:299-319, 1968.

Peltonen J H Larjava, S Jaakkola, H Gralnick, SK Akiyama, SS Yamada, KM Yamada, J Uitto. Localization of integrin receptors for fibronectin, collagen, and laminin in human skin. J.Clin. Invest. 84:1916-1923, 1989.

Potten CS, LP Merkow, M Pardo. Ultrastructural changes in mouse hair follicle pigment cells after plucking, x-rays, and actinomycin D treatments. Lab.Invest. 21:607-616, 1971.

Prota G. Progress in the chemistry of melanins and related metabolites. Med. Res. Rev. 8:525-556, 1988.

Quevedo WC Jr, G Szabo, J Virks. Melanocyte populations in UV-irradiated human skin. J.Invest.Dermat. 45:295-308, 1965.

Rawles ME. Origin of melanophores and their role in development of color patterns in vertebrates. Physiol. Rev. 28:383-408, 1948.

Roberts CJ, TM Birkenmeier, JJ McQuillan SK Akiyama, SS Yamada, WT Chen, KM Yamada, JA McDonald. Transforming growth factor ß stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by culture human lung fibroblasts. J.Biol.Chem. 263:4586-4592, 1988.

Ruoslahti E, FG Giancotti. Integrins and tumor cell dissemination. Cancer Cells. 1:119-126, 1989.

Rusciano D, MM Burger. Why do cancer cells metastasize into particular organs? BioEssays 14:185-194, 1992.

Ryyanen J, S Jaakkola, E Engvall, J Peltonen, J Uitto. Expression of ß4 integrins in human skin: comparison of epidermal distribution with ß1-integrin epitopes, and modulation by calcium and vitamin D3 in cultured keratinocytes. J. Invest. Dermat 97:562-567, 1991.

Sagebiel RW, Odland GF. Ultrastructural identification of melanocytes in early human embryos, in Riley V (ed.): Pigmentation: Its Genesis and Biologic Control New York, Appleton-Century-Crofts. 1972, p 43.

Schroeder HE. Melanin containing organelles in cells of the human gingiva. I. Epithelial melanocytes. J. Periodont. Res. 4:1-18, 1969.

Sonnenberg A, CJT Linders, JH Daams, SJ Kennel. The α6ß1 (VLA-6) and α6ß4 protein complexes: tissue distribution and biochemical properties. J. Cell Sci. 96:207-217, 1990.

Stamp GWH, M Pignatelli. Distribution of ß1, α1, α2, and α3 integrin chains in basal cell carcinomas. J. Pathol. 163:307-313, 1991.

Stern C. Model estimates of the number of gene pairs involved in pigmentation variability of the Negro-American. Hum. Hered. 20:165-168, 1970.

Szabo G. The regional anatomy of the human integument with special reference to the distribution of hair follicles, sweat glands, and melanocytes. Philos. trans. R. Soc. Lond. [Biol.] 252:447, 1967.

Takahashi Y, DF Mutasim, HP Patel, GJ Anhalt, RS Labib, LA Diaz. The use of human pemphigoid autoantibodies to study the fate of epidermal basal cell hemidesmosomes after trypsin dissociation. J.Invest.Dermat. 85:309-313, 1985.

Tamkun JW, DW DeSimone, D Fonda, S Patel, C Buck, AF Horwitz, RO Hynes. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. Cell 46:271-282, 1986.

Tsukamoto K, DM Gersten, LW Law, VJ Hearing. Malignant melanoma: relationship to parameters of differentiation. Melanoma Res. 1:223-230, 1991.

Van Muijaen GNP, LMHA Cornelissen, CFJ Janse, CG Figdor, JP Johnson, EB Brocker, DJ Ruiter. Antigen expression of metastasizing and non-metastasizing human melanoma cells xenografted into nude mice. Clin. Exp. Metastasis 9:259-272, 1991.

Wayner EA, WG Carter, RS Piotrowicz, TJ Kunichi. The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. J.Cell Biol. 107:1881-1891, 1988.

Weiss P, Andres G. Experiments on the fate of embryonic cell (chick) disseminated by the vascular route. J.Exp.Zool. 121:449, 1952.

Wellings SR, Siegel BV. Electron microscopic studies on the subcellular origin and ultrastructure of melanin granules in mammalian melanomas. Ann. NY Acad.Sci. 100:548-568, 1963.

Whittaker JR. Changes in melanogenesis during the dedifferentiation of chick retinal pigment cells in cell culture. Dev. Biol. 8:99-127, 1963.

Whittaker JR. A relationship between increased protein synthesis and loss of melanin synthesis in monolayer cultures of chick retinal pigment cells. Am. Zool. 5:643, 1965.

Zambruno G, V Manca, ML Santantonio, D Soligo, A Giametti. VLA protein expression on epidermal cell (keratinocytes, Langerhans cells, melanocytes): a light and electron microscopic immunohistochemical study. Br. J. Dermat. 124:135-145, 1991.

Zambruno G, PC Marchisio, A Melchiori, S Bondanza, R Cancedda, M DeLuca. Expression of integrin receptors and their role in adhesion, spreading and migration of normal human melanocytes. J. Cell Sci. 105:179-190, 1993.

II. RESEARCH PLAN

RATIONALE

Human Melanocytes and Malignant Melanoma as a Model for Cellular Differentiation and Tumour Progression

The melanocyte is an excellent cell model for studying cellular differentiation and malignant progression *in vivo* [Kerbel, 1992]. Normal epidermal melanocytes reside in the epidermis of the skin, and provide pigmentation and protection from ultraviolet radiation [Tsukamoto et al, 1991]. Melanocytes have several unique phenotypic characteristics that may define the state of cellular differentiation. These include the dendrite formation, slow proliferation rate, the melanin pigment production, and melanosome production. Malignant transformation of the melanocyte results in malignant melanoma. The primary tumours of malignant melanoma arise in the skin, and are visible and easily accessible [Herlyn et al, 1990; Clark et al, 1984].

Tumour progression of malignant melanoma has been classified into three major pathohistological growth phases, which have biological and clinical significance [Clark et al, 1984]. The first phase is exemplified by an increase in melanocyte growth and migration along the basement membrane within the epidermis in a radial pattern, without invasion of the underlying dermis. Four clinical and pathohistological subtypes are recognized, based upon the morphology and nesting of the melanocytes. This first growth phase is called the radial growth phase (RGP). The second growth phase is identified by the appearance of melanocytes within the underlying dermis. The tumour is thicker than the epidermis, and takes on a more vertical orientation. Melanocytes in the dermis appear different than the epidermal melanocytes, with loss of some cellular differentiation phenotype compared to the epidermal melanocytes. This phase is called the vertical growth phase (VGP). The last phase is identified by the presence of secondary metastatic colonies of melanoma cells in distant organs. The melanoma cells in these metastatic colonies have nuclear atypia, and are heterogenous in cytoplasmic content. The state of cellular differentiation is quite variable, but appears to be less differentiated than the original tumour cells [Tsukamoto et al, 1991]. However, attempts to correlate tumour growth with differentiation *in vivo* have been confounded by the heterogeneity displayed by tumour cells [Albino 1992; Lynch et al, 1991]. This third growth phase is related to the metastatic spread of melanoma, hence called the metastatic growth phase (MGP).

In an attempt to minimize tumour heterogeneity, Herlyn and associates [1987] have established a bank of melanoma cell lines derived from melanoma tumours representing the three different phases of tumour progression [Clark et al, 1984]. Thus, it now may be possible to study cellular and molecular characteristics of cells representing each stage of tumour progression *in vivo*. However, the characterization of the established cell lines has not been fully performed, nor have the association between phenotypic expressions of *in vitro* differentiation and *in vivo* tumour progression been elicited.

The association between phenotypic expression of *in vitro* differentiation and *in vivo* malignant tumour progression can be studied by altering the differentiation state of cell lines using agents which can reversibly change the differentiation phenotype [Albino, 1992; Tsukamoto et al, 1991]. One example is a thymidine analogue, 5-bromo-2-deoxyuridine (BRDU) [Thomas et al, 1993]. BRDU substitutes for thymidine in DNA, causing sister chromatid exchanges in fragile regions which are higher than expected statistical frequency [LeBeau et al, 1986]. These regions frequently contain critical regulatory genes responsible for differentiation phenotype [Valyi-Nagy et al, 1993]. Thus, BRDU can influence the differentiation state in many malignant cells *in vitro* [Valyi-Nagy et al, 1993]. Furthermore, these changes have been shown to alter the differentiation phenotypes of cells, and even eliminate some markers unique for malignancy, such as cell migration, and mitosis [Thomas et al, 1993; Tapscott et al, 1989]. A second example is the α-melanocyte stimulating hormone (α-MSH) [Sawyer et al, 1980], which causes an increase in melanin pigmentation *in vivo* and *in vitro*, with a decrease in proliferative capacity and tumourigenic potential of cells [Preston et al, 1987]. Thus, these two agents can alter the differentiation phenotype of melanocytes through different mechanisms. However, it is not known how BRDU or α-MSH can affect the phenotypic differentiation of malignant melanoma cells derived from different phases of tumour progression.

Malignant Progression in Melanoma and Integrin-Mediated Cell-Substrate Adhesion

Histopathologically, there are three major biological events that provide different stages of melanoma tumour progression, and each is marked by an alteration in cell-substrate adhesion. The first is the appearance of melanocytes above the basal layer of the epidermis [Clark et al, 1984; Clark, 1991]. Since normal melanocytes are always located on the basement membrane [Clark, 1991], the loss of attachment to the basement membrane by the transformed melanocyte during the radial growth phase may indicate a loss of adhesion to the basement membrane components [Kerbel, 1992]. During the vertical growth phase, the melanoma cells still maintain adherance of individual cells, but initiate migration on extracellular matrices which are not found within the normal epidermal basement membrane. Thus, these cells acquire the components which enable adhesion and migration over novel extracellular matrices. Clinically, this event marks the evolution of a true malignancy, and prognostically, the survival rate is lower [Liotta et al, 1991]. During the metastatic phase, the melanoma cells have the capacity for migrating into and then spread out of the circulation, disseminating to an environment much different from the normal epidermis. They now form new tumours [Liotta et al, 1991]. Clinically, this stage carries a poor prognosis, with the death of the host occuring within weeks to months [Liotta et al, 1991]. While each of these threshold events can be recognized clinically and histopathologically, very little is known at the cellular and molecular level as to how melanoma cells adhere to this new environment at each of these stages [Tsukamoto et al, 1991]. A better understanding of how cells change their adhesion to extracellular matrices may identify critical events underlying malignant progression, and how such changes lead to the increasingly aggressive behaviour from RGP to MGP by individual tumour cells.

Cells utilize cell surface molecules for recognizing and attaching to extracellular matrices. The major cell-substrate adhesion molecule family is the integrin family, a α-β heterodimer which links the extracellular matrix with the cell cytoskeleton [Marchisio et al, 1991]. There are multiple α and β subunits, each capable of combining into a heterodimer prior to surface expression [Hynes, 1992]. This diversity makes the integrin an ideal candidate for modulating cell adhesion. It is not known whether melanoma cells derived from different phases of malignant progression can alter the expression of integrins on the cell surface, or whether changes in the phenotypic expression of differentiation would also change integrin expression.

Recent studies on lymphocyte homing indicate a role for integrins in cell emigration from the bloodstream. Integrin affinity for ligand can be greatly increased by prior activation through other cell adhesion molecules (i.e., selectins and chemoattractants) [Springer, 1994]. Thus, it is possible that metastatic melanoma cells may be able to regulate integrin activation via other factors, which would enable emigration of cells from the bloodstream, at a site containing a suitable growth environment. It is not known how malignant cells may regulate the integrin affinity for substrates in this manner.

Integrin-ligand binding is dependent on the presence of divalent cations in the extracellular space, which occupy an "EF-hand-like" binding site on the α-subunit [Loftus et al, 1990]. Thus, occupancy of the cation binding site by a divalent cation produces a favourable binding conformation of the integrin heterodimer. Different divalent cations can alter the conformational state, and increase the binding affinity [Grzesiak et al, 1992]. This activation alone can cause changes in cell morphology and behaviour, through some unknown signalling mechanism [Grinnell and Backman, 1991].

Cell lysis can release intracellular cations into the extracellular space, where integrins expressed on neighbouring cells may be affected. Clinically, tumours which undergo necrosis and ulcerate are more aggressive than tumours which do not ulcerate [Clark, 1991]. It is not known whether melanoma cells from different phases of malignant progression could take advantage of different divalent cations to alter cell-substrate adhesion.

While cell-substrate adhesion is dependent upon the interaction between extracellular matrices and integrins, it also requires the interaction between the integrin and the cell cytoskeleton [Akiyama et al, 1989; Mueller et al, 1989]. Cytoskeletal organization can change with tumour progression, and also with changes in differentiation phenotype [Hynes, 1992]. Thus, changes in cell-substrate adhesion could be attributed to changes within the cytoskeletal organization [Hynes, 1992]. Organization of the cytoskeleton is also important for cell migration, and even regulation of cytoplasmic and nuclear control of gene expression [Ingber, 1993]. It is not known whether changes in cytoskeletal organization occurs in melanoma cells derived from different phases of tumour progression, or whether changes could be associated with differences in differentiation phenotype. What remains to be defined may include whether phenotypic expression of cellular differentiation *in vitro* can be linked to malignant tumour progression *in vivo*; and whether the ability of cells to adhere to either whole extracellular matrix components, or adhesion peptides can be also linked to cellular differentiation *in vitro* or malignant tumour progression *in vivo*. These processes may be brought about through alterations in integrin expression, or by integrin interactions with either the extracellular matrix, or the cytoskeleton.

OBJECTIVES AND HYPOTHESES

In order to determine whether there is any relationship between phenotypic expression of cellular differentiation and tumour progression phases, I will test the following hypotheses:

1) Normal human melanocytes and melanoma cells may have a common/or unique phenotypic expression of differentiation markers according to the growth phase of tumour progression.

2) The administration of differentiation-inducing agents, BRDU and α-MSH, may alter this phenotypic expression of differentiation markers by normal and malignant melanocytes,

depending on the growth phase of tumour progression.

In order to determine whether integrin-mediated cell-substrate adhesion is related to cellular differentiation and tumour progression, I will test the following hypotheses:

3) The phenotypic expression of integrin subunits on normal and malignant human melanocytes will differ with the state of cellular differentiation and/or the growth phases of tumour progression. Furthermore, there would be different interactions between integrin subunit expression and cell-substrate adhesion to whole extracellular matrix molecules among melanoma cell lines derived from different growth phases.

4) The administration of BRDU and α-MSH may alter the interaction between integrin subunit expression and integrin-mediated cell-substrate adhesion, based upon the changes in the state of cellular differentiation and the growth phases of tumour progression.

EXPERIMENTAL STRATEGY

In Chapter III, the differences in the major phenotypic differentiation markers between normal and malignant melanocytes under defined growth phases from representative stages of tumour progression will be characterized. Specifically, the major melanocyte differentiation markers (e.g., melanin pigmentation, tyrosinase activity, cell morphology, and proliferation rate) will be compared between normal human epidermal melanocytes and human melanoma cells derived from tumours representing specific growth phases of malignant transformation. In addition, these cellular differentiation markers will be compared between normal and malignant melanocytes exposed to BRDU, or α-MSH, which should affect cellular differentiation of both normal and malignant cell lines. The use of differentiation-inducing agents within the same cell line can also minimize the phenotypic heterogeneity observed in individual culture cell lines. This comparison will determine whether phenotypic expression of cellular differentiation correlates with the growth phases of malignant progression.

Chapter IV will deal with the changes in the integrin expression and function with respect to (i) normal and malignant growth phases of melanocytes, and (ii) normal and malignant melanocytes cultivated in the presence of the reversible differentiation-inducing agents, BRDU and α-MSH. The degree of integrin surface expression by normal and malignant human melanocytes will be measured by flow cytometry using commercially available antibodies against integrin subunits. Differences in integrin expression will be compared, in order to determine whether integrin expression is associated with (i) the different growth phases of tumour progression, and (ii) cellular differentiation phenotype.

In Chapter V, the short-term adhesion to extracellular matrix components will be compared between normal and malignant human melanocytes by using a quantitative cell adhesion assay [Lotz et al, 1991], in order to determine whether differences in cell-substrate adhesion is associated with (i) the different growth phases of tumour progression, and (ii) cellular differentiation phenotype.

In Chapter VI, short-term adhesion to whole extracellular matrix components in the presence of functional blocking antibodies and divalent cations will be compared between normal and malignant human melanocytes by using the quantitative cell adhesion assay, in order to determine whether sensitivity of integrins for activation through divalent cations is associated with (i) the different growth phases of tumour progression, and (ii) cellular differentiation phenotype.

In Chapter VII, the findings of the above four chapters will be summarized together, illustrating how malignant melanoma cells alter their ability to use integrins for cell-substrate adhesion during malignant progression. In addition, the effect of differentiation state on integrin-mediated cell-substrate adhesion will be summarized. This comparison will determine whether integrin expression and its role in cell-substrate adhesion correlates with tumour progression, and/or state of cellular differentiation.

SIGNIFICANCE

Malignant melanoma is an excellent cell model for exploring the effects of cellular differentiation on tumour progression. Changes in cell-substrate adhesion appears to be an integral component in tumour progression, and the acquisition of the fundamental qualities of malignancy, namely, invasion and metastasis. The results are expected to give new insights into cell-substate interactions and thus will provide a basis for better understanding how normal melanocytes attach to the basement membrane, and how their neoplastic counterparts, melanoma cells dissociate from and migrate through the basement membrane into the underlying stroma and to distant organs. This may help us in designing more rational clinical approaches to prevent or control the melanoma invasion and metastasis.

BIBLIOGRAPHY

Akiyama SK, SS Yamada, WT Chen, KM Yamada. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. J.Cell Biol. 109:863-75, 1989.

Albino AP. The role of oncogenes and growth facators in progressive melanoma-genesis. Pigment Cell Res. Suppl 2:199-218, 1992.

Clark WH. Tumor progression and the nature of cancer. Br.J.Cancer, 64:631-644, 1991.

Clark WH, DE Elder, D Guerry, MN Epstein IV, MH Greene, M Van Horn. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. Hum. Pathol. 15:1147-1165, 1984.

Grinnell F, R Backman. Role of integrin receptors in manganese-dependent BHK cell spreading on albumin-coated substrata. Exp.Cell Res. 195:218-223, 1991.

Grzesiak JJ, GE Davis, D Kirchhofer, MD Pierschbacher. Regulation of $\alpha2\beta1$-mediated fibroblast migration on type I collagen by shifts in the concentrations of extracellular Mg^{+2} and Ca^{+2}. J.Cell Biol. 117:1109-1117, 1992.

Herlyn M, Clark WH, Rodeck U, Mancianti ML, Jambrosic J, Koprowski H. Biology of tumor progression in human melanocytes. Lab.Invest. 56:461-474, 1987.

Herlyn M. Human melanoma: development and progression Cancer Metas.Rev. 9:101-112, 1990.

Hynes RO. Integrins: verstility, modulation, and signalling in cell adhesion. Cell 69:11-25, 1992.

Ingber DE. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. J.Cell Sci. 104:613-627, 1993.

Kerbel RS. Expression of multi-cytokine resistance and multi-growth factor independence in advanced stage metastatic cancer. Am. J. Pathol. 141:519-524, 1992.

LeBeau MM. Chromosomal fragile sites and cancer-specific rearrangements. Blood 67:849-858, 1986.

Liotta LA, WG Stetler-Stevenson, PS Steeg. Cancer invasion and metastasis: positive and negative regulatory elements. Cancer Invest. 9:543-551, 1991.

Loftus JC, TE O'Toole, EF Plow, A Glass, AL Frelinger III, MH Ginsberg. A ß3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. Science 249:915-8, 1990.

Lotz MM, CA Burdsal, HP Erickson, DR McClay. Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. J.Cell Biol. 109:1795-1805, 1989.

Lynch SA, BN Bouchard, S Vijayasanadhi, H Yuasa, AN Houghton. Antigens of melanocytes and melanoma. Cancer Metastasis Rev. 10:141-50, 1991.

Marchisio PC, S Bondanza, O Cremona, R Cancedda, M DeLuca. Polarized expression of integrin receptors (α6ß4, α2ß1, α3ß1, and αvß3) and their relationship with the cytoskeleton and basement membrane matrix in cultured human keratinocytes. J.Cell Biol. 122:761-73, 1991.

Mueller SC, T Kelly, MZ Dai, HN Dai, WT Chen. Dynamic cytoskeleton-integrin associations induced by cell binding to immobilized fibronectin. J. Cell Biol. 109:3455-64, 1989.

Preston SF, M Volpi, CM Pearson, RD Berlin. Regulation of cell shape in the Cloudman melanoma cell line. Proc.Natl.Acad.Sci.(USA) 84:5247-5251, 1987.

Sawyer TK, PJ Sanfilippo, VJ Hruby, MH Engel, CB Heward, JB Burnett, ME Hadley. 4-Norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone: a higly potent α-melanotropin with ultralong biological activity. Proc.Natl.Acad.Sci.(USA) 77:5754-5758, 1980.

Springer TA. Traffic signals for lymphocyte recirculation and leucocyte emigration: the multistep paradigm. Cell 76:301-14, 1994.

Tapscott SJ, AB Lassar, RL David, H Weintraub. 5-bromo-2'-deoxyuridine blocks myogenesis by extinguishing expression of Myo D1. Science 245:532-536, 1989.

Thomas L, PW Chan, S Chang, C Damsky. 5-bromo-2-deoxyuridine regulates invasiveness and expression of integrins and matrix-degrading proteinases in a differentiated hamster melanoma cell. J. Cell Sci. 105:191-201, 1993.

Tsukamoto K, DM Gersten, LW Law, VJ Hearing. Malignant melanoma: relationship to parameters of differentiation. Melanoma Res. 1:223-230, 1991.

Valyi-Nagy, I, I-M Shih, T Gyorfi, D Greenstein, I Juhasz, DE Elder, M Herlyn. Spontaneous and induced differentiation of human melanoma cells. Int. J. Cancer 54:159-165, 1993.

III. GROWTH AND DIFFERENTIATION CHARACTERISTICS OF NORMAL HUMAN MELANOCYTES AND MELANOMA CELLS DERIVED FROM DIFFERENT PHASES OF TUMOUR PROGRESSION AFTER EXPOSURE TO 5-BROMODEOXYURIDINE AND α-MELANOCYTE STIMULATING HORMONE[*]

INTRODUCTION

Tumour progression involves a series of multi-step events in which a putatively normal cell proceeds to a metastatic cancer cell. This tumour progression may further be divided into two phases on the basis of growth characteristics; (a) "restricted" (not autonomous), and (b) "unrestricted" (autonomous). The melanocytic tumour appears to provide an excellent model for the study of biological processes underlying the "restricted" and "unrestricted" stages of tumour progression. This is based upon clinical and histopathological observations of malignant melanoma which show well defined stages of tumour progression. The first "restricted" stage of tumour progression in malignant melanoma is the radial growth phase (RGP), wherein a primary melanoma grows in the epidermis without any invasion to the basement membrane. The next stage is the vertical growth phase (VGP), wherein melanoma cells become "semi-restricted" and begin to grow downward beyond the basement membrane. In both RGP and VGP phases, the melanoma cells exhibit unique differentiation features which are distinct from the putative normal melanocyte. These unique features are manifested by a number of biological properties and differentiation phenotypes including cell morphology, pigmentation, cell proliferation, and expression of specialized proteins [Houghton et al, 1987]. Previous studies have relied on cultivation of cells from tumours at various phases of tumour progression *in vivo*. Herlyn has been the most successful in this regard, collecting many stable tumour cell lines from tumours at all phases of malignant progression [Herlyn et al, 1985a].

Malignant melanoma rarely involutes into a benign tumour through spontaneous terminal differentiation [Clark et al, 1984]. Spontaneous differentiation is part of the natural history of benign nevi [Aso et al, 1988]. Thus, *in vivo* evidence suggests that malignant progression may be a multifactorial process of dedifferentiation. Metastatic malignant cells lose the growth restriction characteristics of their normally differentiated or "restricted" counterparts [Tsukamoto et al, 1991; Albino, 1992; Wollina et al, 1991; Valyi-Nagy et al, 1993]. In general the cells in this "unrestricted" stage reveal less differentiated phenotypes. The terminal-differentiation-inducing agents, BRDU and α-MSH, may change the differentiation and growth states of melanoma cell lines. It is interesting to know how such differentiation-inducing agents would affect the differentiation phenotypes of cell lines derived from "restricted" stages of melanoma progression. This study is an attempt to firstly compare the growth behaviour and the expression of differentiation phenotypes between normal human melanocytes and human melanoma cells from six cell lines that were derived from three major growth phases of malignant melanoma progression. Secondly, the changes in their growth patterns and phenotypic expressions are determined following exposure to either 5-bromo-2'-deoxyuridine (BRDU); or [4-Norleucine,7-Phenylalanine]-α-melanocyte stimulating hormone {[Nle^4,D-Phe^7]-α-MSH}, a potent, synthetic analogue of α-melanocyte stimulating hormone (α-MSH) [Sawyer et al, 1980], two common differentiation-inducing agents used with melanocytes.

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[*] **A version of this chapter has been submitted for publication to International Journal of Cancer.**

MATERIALS AND METHODS

Materials:

Culture media, RPMI 1640 medium, F-10 medium, Trypsin/EDTA, penicillin/streptomycin/amphotericin B, was obtained from Gibco (Mississauga, ON). L-Dopa, phorbol-12,13-dibutyrate, 5-bromo-2'-deoxyuridine, sulforhodamine B, trichloroacetic acid, uranyl acetate, and lead citrate were obtained from Sigma (St. Louis, MO). Glutaraldehyde (EM Grade), and osmium tetroxide were obtained from JBEM (Pointe-Claire, Dorval, PQ). Deoxycholate (DOC) and sodium docedyl sulfate (SDS) were obtained from Boeringer Mannheim (Toronto, ON), 4-Norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone ([Nle^4,D-Phe^7]-α-MSH) was obtained from Peninsula Labs (Belmont, CA). Fetal bovine serum was purchased from Upstate Biologicals Inc. (Rochester, NY). Bovine pituitary extract was purchased from Clonetics (San Diego, CA). Cells were cultivated in either 20 cm^2 tissue culture plates (Sarstedt, Toronto, ON); or 96-microwell plates (Nunc, Mississauga, ON). Sodium hydroxide and acetic acid (glacial) were obtained from BDH (Edmonton, AB). Propylene oxide was obtained from Aldrich (Milwakee, WI). Tris base was obtained from ICN (Mississauga, ON). Collagen Type I was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Nonidet P-40 was obtained from United States Biochemicals (Cleveland, OH).

Cell Lines:

Normal human epidermal melanocytes were cultivated from primary cultures of human neonatal foreskin using the method of Eisinger and Marko [1982] with some modifications. These included the addition of bovine pituitary extract to F-10 media, and the utilization of a 4-fold increased concentration of phorbol-12,13-dibutyrate (PdBU) to 2 μg/ml.

Six malignant melanoma cell lines were used: WM 35 (radial growth phase) and G 361 (metastatic growth phase) were obtained from the American Type Culture Collection (ATCC; Rockville, MD), WM 39, WM 902B, and WM 1341D (vertical growth phase) were generously provided by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA). SK-MEL 23 (metastatic growth phase) was generously provided by Dr. A Houghton (Memorial Sloan-Kettering Institute, Rochester, NY).

All melanoma cell lines were cultivated in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and with penicillin/streptomycin/amphotericin B added as per manufacturer's instructions. Media was replaced every 3 days. Cell passage was performed with trypsin/EDTA.

For differentiation-induction with BRDU, a 120 μl aliquot of BRDU (50 mM in DMSO) was added to 500 ml of complete medium (final concentration, 18 μM). Cells were passaged if the cell confluency was approaching 90%. For differentation-induction with α-MSH, [Nle^4,D-Phe^7]-α-MSH (10^{-4} M) was added to the complete medium to a concentration of 10^{-7} M. The medium was replaced every 3 days, and cells were harvested for assays after 7 days induction.

Cell Morphology Studies:

Light microscopy was performed by photographing unfixed cells using a Nikon inverted microscope at 200 x magnification under phase contrast optics, on Kodak 2415 Black and White film (ASA 32).

Electron microscopy was performed by fixing the cells grown on the culture plate with 2.5% glutaraldehyde for 15 minutes. Cells were gently scraped using a rubber policeman, pelleted, and post-fixed with 1% osmium tetroxide in phosphate buffer for 60 minutes. The pellet was sequentially dehydrated with increasing grades of ethanol, cleared with propylene oxide for 15 minutes, embedded overnight in a propylene oxide/epoxy resin mixture (1:1) at room temperature, placed in pure epoxy resin under vacuum for 24 hours, and then incubated at 50°C for 3 days. Thin sections were made by a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with a Philips 300 transmission electron microscope. Comparisons of ultrastructural morphology between cell lines were examined by two independent investigators and a consensus was achieved.

Cell Proliferation Assay:

Cells were plated into wells of 96-well plates at a density of $5x10^3$ cells/100μl of the appropriate media, and the cell numbers were determined at 0, 24 and 48 hours by measuring protein using the colourimetric sulforhodamine B assay of Skehan and colleagues [1990]. Briefly, cells were fixed by adding 20 μl of 50% trichloroacetic acid in PBS (pH 7.4) at 4°C for one hour. The wells were washed three times with running tap water and allowed to air dry. Fifty μl of 0.4% sulforhodamine B in 1% acetic acid was added to each well and incubated at room temperature for 30 minutes. The plates were washed by immersion 4 times with 1% acetic acid and air dried. The colourimetric reaction was performed by adding 50 μl of 10 mM Tris-base (pH 10.0) to each well and incubating at room temperature on a rotary table for 5 minutes. The absorbance was read at 450 nm with a microplate reader (SLT Industries, Salzberg, Austria). After correcting for the blank absorbance, the proliferation rate was calculated according to the formula: $A_t = A_o e^{lt}$, where A_t is the absorbance at time, t; A_o is the absorbance at time, 0; and $l = 0.693/t_2$, t_2 being the doubling time, in hours. Statistical significance was determined by paired Student t-determination to compare differences between experimental and control groups.

Soft Agar Colony Formation Assay:

Formation of colonies in soft agar was determined using the method of Bouck and DiMayorca [1977]. Briefly, for each cell line, five 60 mm plates were prepared. Five ml of complete agar medium was casted for each 60 mm plate, and allowed to solidify at room temperature. Cells were harvested and suspended in medium at a density of 10^6 cells/ml. A mixture of 5.5 ml of cell suspension with 11 ml of complete agar medium prepared and kept at 45°C. was made, and aliquoted at 1.5 ml per plate, to give a final cell number of $5x10^4$ cells per plate, and a final agar concentration of 0.34%. Control liquid cloning ability was tested by suspending $2x10^2$ cells in 1.5 ml of liquid medium (10 μl of cell suspension in 20 ml medium, plating 3 ml/plate) over a soft agar surface. Cells were incubated for 7 days in the case of the liquid cloning assay, and 18 - 21 days for the soft agar assay. The number of visible colonies (> 0.2 mm diameter) was counted without staining. Determination of the cloning efficiency was calculated by the ratio of (colony number - liquid cloning number)/(number of cells per plate initially plated) x 100. Values from replicate plates were combined and the mean and standard deviation were determined. Statistical significance was determined by paired Student t-determination to compare differences between experimental and control groups.

Cell Migration Assay:

Nunc Tissue Culture inserts (10 mm dia, 8.0 μm pore size) were placed into wells (24-well plate, Nunc) containing 300 μl of solution consisting of 10 μg/ml collagen type I in media, and incubated for 1 hr at 37°C. After rinsing with PBS, the membrane was blocked with 5% nonfat milk in PBS for 30 min at 37°C. The membrane was rinsed again with PBS, and placed in wells containing 600 μl of media containing 10 μg/ml collagen type I.

Cells were labelled using di-O (10 μg/ml; Molecular Probes) in media for 2 hr at 37°C. Cells were washed twice in PBS, and harvested using 10 mM EDTA in PBS. Cell suspensions (5×10^4/100 μl) were placed in the upper chamber and incubated at 37°C. for 6 hr. Cells remaining in the upper chamber were removed using a cotton-tipped applicator. The membrane was placed into 600 μl of 1% (v/v) Nonidet P (NP)-40 in PBS, and an additional 100 μl of 1% NP-40 was added to the upper chamber and incubated at 37°C. for 15 min. The solution in the upper chamber was combined with the lower chamber, and the fluorescence intensity was determined on a spectrofluorometer (SPEX; Canberra Packard, Toronto, ON) using an excitation wavelength at 488 nm, and a emission wavelength at 501 nm. Percent migration was determined by the ratio between the measured fluorescence and the fluorescence of a dissolved cell pellet containing 5×10^4 cells. Statistical significance was determined by paired Student t-determination to compare differences between experimental and control groups.

Cellular Tyrosinase Activity:

The dopa oxidase activity of human tyrosinase was determined by using the method of Whittaker [1981]. Cells were harvested using 10 mM EDTA in PBS and washed once in PBS (pH 7.4). Cells were suspended in PBS (pH 6.8) at a density of 2.67×10^5 cells/ml. Seventy-five μl of cell suspension was seeded into each well of a 96-microwell plate. An additional 75 μl of 0.2 M L-Dopa in PBS (pH 6.8) was added to the wells. The plate was incubated at 37°C. for 3 hrs. Blank wells contained 150 μl of 0.1 M L-DOPA in PBS (pH 6.8) only. Optical absorbance at 405 nm was measured on a microplate reader (SLT Industries, Salzberg, Austria), and standardized to 10^6 cells. Means and standard deviations of 8 wells were determined. Statistical significance was determined by paired Student t-determination to compare differences between experimental and control groups.

Cellular Melanin Content:

Cells were harvested using 10 mM EDTA in PBS and washed once in PBS (pH 7.4). Cells were suspended in PBS (pH 6.8) at a density of 2.67 x 10^5 cells/ml. Seventy-five µl of cell suspension was seeded into each well of a 96-microwell plate. An additional 75 µl of 10 N NaOH was added to each well. The plate was incubated at 68°C. for 16 hrs. Optical absorbance at 405 nm was measured on a microplate reader (SLT Industries, Salzberg, Austria),and standardized to 10^6 cells. Means and standard deviations of 8 wells were determined. Statistical significance was determined by paired Student t-determination to compare differences between experimental and control groups.

Tyrosinase-Related Protein-1 Assay:

Cells were placed in methionine-free RPMI 1640 for one hr. Thirty µl of [^{35}S]-methionine (250 µCi/ml) (Amersham, Oakville, ON) was added to each 150-cm² plate, and incubated for 16 hrs. at 37°C. Cells were washed three times in PBS (pH 7.4), and lysed in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris (pH 8.0)] at 4°C. for 30 min. Aliquots of 1.5 ml were placed in Eppendorf tubes and centrifuged at 10,000xg for 15 min. The supernatant was precleared with normal mouse serum-agarose and Protein A-Sepharose prior to immunoprecipitation with 25 µl of an anti-human tyrosinase related protein (TRP)-1 using a monoclonal antibody (MoAb) HMSA-5 for 4 hr [Der et al, 1993]. This murine antibody was bridged using 10 µl of goat anti-mouse immunoglobulin, and precipitated with 50 µl Protein A-Sepharose. After washing, the pellet was boiled for 10 min. in reducing sample buffer and separated on a 15% SDS-PAGE according to the method of Laemmli [1970]. The gel was fixed, and incubated in pure DMSO for 30 min, followed by 22% PPO/DMSO for 30 min, and washed in tap water. After drying the gel, it was exposed to Kodak X-Omat film for 16 hr at -70°C. The film was developed according to manufacturer's instructions. The exposed film was digitized (NIH Imager, using an Apple Color Onescanner), and the optical density of each band was determined.

RESULTS

Cell Morphology by Light Microscopy

On examination with light microscopy, four parameters were considered for the measurement of cell differentiation [Houghton et al, 1987]. These four parameters were further divided into two groups; the cell perikaryon (size and shape), and the formation of dendrites (number per cell and dendrite length). The perikaryon shape was classified as one of three shapes: neuronal (spindle shape with slender cell hillock), fibroblastic (polygonal with broad cytoplasmic extensions), or epithelioid (rectangular with no significant cytoplasmic extensions). Perikaryon size of treated cells was compared to control, and was considered to be larger or smaller than the control surface area. Dendrites are cytoplasmic extensions from the perikaryon, and have a very small cross-sectional diameter. The average number of dendritic extensions was determined for each cell. Also, the length of the dendrite (compared to cell body diameter) from the perikaryon was compared. Cells expressing a less differentiated cell-membrane antigen phenotype (according to Houghton [1987]) had an epithelioid morphology with few, short dendrites, or no dendrites at all.

The majority of the untreated control cell lines had a small neuronal (spindle-shaped) perikaryon with two to three dendrites extending one to two cell lengths (Figure III-1). The exceptions were WM 39 (RGP), which had a large fibroblastic morphology and few, short stubby dendritic extensions; and the melanoma cell lines WM 1341D (VGP) and SK-MEL 23 (MGP), which had a large epithelioid morphology with no dendrites. There was some phenotypic heterogeneity within all cell lines, but the predominant morphology was selected as a representative for the cell line. There were no consistent trends in either the perikaryon shape or size between normal and malignant cells as a function of the growth phase of tumour progression. Houghton and colleagues [1987] found that melanoma cells which had a neuronal perikaryon with many long dendrites expressed more mature differentiation cell-membrane antigens.

The effect of differentiation-inducing agents on cell phenotype was examined. The treatment with α-MSH did not change the perikaryon shape of cell lines with a baseline morphology (Normal melanocytes, WM 35, WM 902B, SK-MEL 23, and G 361), although the cell size tended to become larger, and more and longer dendrites were observed (Figure III-1). The WM 1341D cell line with an epithelioid morphology became more fibroblastic in shape with longer dendrites, but without a significant change in cell size. However, treatment with BRDU caused several distinctive changes in cell morphology for all cells, except normal melanocytes. The radial growth phase WM 35 cell line developed a larger, epithelioid perikaryon with an increase in dendrite number on some cells. The vertical growth phase WM 39 cell line increased the size of the fibroblastic perikaryon shape without changing the observed number or size of dendrites. The VGP WM 902B cell line and the metastatic growth phase G 361 cell line maintained their neuronal perikaryon shape, but increased the dendritic length.

However, the most dramatic change was observed with the VGP WM 1341D, which changed from an epithelioid to a neuronal perikaryon morphology, with a great increase in longer dendrites. Taken together, these findings suggest that all transformed cells are sensitive to the differentiation-inducing agents, BRDU and α-MSH, and that BRDU tend to produce cell features consistent with a less differentiated phenotype, while α-MSH produce the opposite changes. The lone exception to this trend was the VGP WM 1341D, which developed features of a more differentiated phenotype with BRDU. Thus, the regulatory mechanisms responsible for differentiation features could be influenced by these reversible differentiation-induction agents.

Cells which were cultivated in the presence of differentiation-inducing agents and then further cultivated in control media were observed to return to the control phenotype after 7 days. Thus, the effect of these agents did not induce a permanent alteration in the cellular phenotype at the light microscopic level.

Cell Morphology by Electron Microscopy

Randomly selected electron micrographs of each cell line was examined by two independent investigators. Differences in ultrastructural morphology were compared between untreated cells and those treated with differentiation-inducing agents. Before exposure to differentiation-induction agents, the major phenotypic parameter of interest in the electron microscopy studies was the degree of melanosome development. Melanosomes progress through 4 structural stages during maturation [Hirobe, 1992]. The Stage I melanosome is a round, membrane-lined intracellular vesicle about 0.5 μm in diameter, with a scant lamellar interior. Stage II melanosomes are ellipsoidal in shape, and show lamellar structures with regular striation. Melanin deposition begins in Stage III, and IV melanosomes and show increased melanin deposits to an extent that the lamella are completely obliterated. Only the Stage IV melanosome has been demonstrated in keratinocytes, implying that less mature melanosomes lack a surface molecule which permits the melanocyte to transfer the melanosome into the surrounding cells. Therefore, the degree of maturation of melanosomal structure directly correlates with melanization, which is a sign of increased differentiation.

Normal melanocytes had mainly ellipsoidal, completely melanized Stage IV melanosomes which were individually dispersed throughout the cytoplasm (data not shown). The RGP MW 35 (Figure III-2a-c), and the VGP WM 1341D (Figure III-4a-c) and WM 39 (Figure III-3a,b) cells had individually-dispersed, ellipsoidal, incompletely melanized Stage III melanosomes scattered throughout the cytoplasm. No autodegradation was observed in any melanosome. However, both the MGP SK-MEL 23 (Figure III-5a) and G 361 (Figure III-5e) cells had Stage III melanosomes which were more round, or oval, and each cell line had a variable number of autodegraded Stage III melanosomes, implying that the structural matrix of the melanosomes was incomplete, or abnormal. Only the SK-MEL 23 cell line had melanosomes which were completely melanized (Stage IV) (Figure III-5a). Thus, while the changes in melanosome morphology was not dramatic between RGP, VGP, and MGP cells, the more rounded morphology in conjunction with increased autodegradation would suggest that the melanosomal morphology was less mature in the later phases of tumour progression.

After exposure to BRDU and α-MSH, the RGP WM 35 (Figure III-2d,e), the VGP cell lines WM 1341D (Figure III-4d,e) and WM 39 (Figure III-3c-e), and the MGP SK-MEL 23 (Figure III-5b) cell lines showed a significant change in melanosome morphology following treatment with BRDU and α-MSH. BRDU treatment did not have any effect on melanosome morphology with normal melanocytes. However, BRDU treatment of WM 35 cells caused a decrease in melanosome number, and their shape became round and much less melanized (Figure III-2d,e). Additionally, there was significant autodegradation of melanosomes, indicating a less stable melanosome matrix. BRDU treatment of WM 39 (Figure III-3c,d) cells led to a decrease in melanosome number, but did not change the degree of melanization, nor were any autodegraded melanosomes detected. SK-MEL 23 cells had the most dramatic alteration, with replacement of Stage IV melanosomes by fewer Stage II melanosomes (Figure III-5b). In contrast, BRDU treatment of the VGP WM 1341D cells greatly increased the number of melanosomes, and a greater proportion of the melanosomes were the mature Stage IV type (Figure III-4d,e). This finding indicated that BRDU treatment of WM 1341D cells produced a more mature melanosome morphology, which was contrary to the decreased maturation by melanosomes observed with all other melanoma cell lines. Since maturation of melanosomes directly correlated with the state of differentiation, BRDU treatment of melanoma cells, except WM 1341D, produced a cell line which exhibited a less differentiated phenotype.

Only WM 35 (Figure III-2f,g) and WM 39 (Figure III-3f) cell lines had some unique changes in melanosome morphology following α-MSH treatment. Both cell lines had an increase in melanosome number and proportion of Stage IV melanosomes following treatment. This finding indicated that α-MSH caused a maturation of melanosomes, which appeared to develop a more differentiated phenotype.

Therefore, changes in cell morphology, and in melanosomal maturation after exposure to the differentiation-induction agents indicated that the state of differentiation decreases with tumour progression. Furthermore, treatment with BRDU caused a decrease in melanization and a more epithelioid cell morphology, the lone exception being the VGP melanoma cell line WM 1341D, which showed an increase in melanization and a more spindle cell morphology. Treatment with α-MSH increased melanization, but did not affect cell shape in the WM 35 and WM 39 cell lines.

Cell Proliferation

Before treatment with differentiation-induction agents, all malignant cell lines had shorter doubling times, compared with normal melanocytes (Figure III-6). No appreciable differences in doubling rates could be determined between malignant cell lines derived from three different phases of tumour progression, except for the RGP WM 1341D. The doubling time for this cell line was intermediate between that of normal melanocytes and the other melanoma cells from the radial, vertical and metastatic growtn phases. Thus, a decrease in doubling rates could distinguish between normal and malignant cells, but not between malignant cells of different phases of tumour progression.

After exposure with differentiation-induction agents, most malignant cell lines had a doubling time of about 10 to 12 hours, which did not change with induction by either BRDU or α-MSH (Figure III-6). However, with BRDU, the doubling time for WM 902B increased from 13 hr to 26 hr. This increased doubling time for cell proliferation, also reported for other cell lines, implies that the intercalation of BRDU into the cellular DNA increases the synthesis phase of the cell cycle [Morris et al, 1989].

Soft Agar Colony Formation

Before treatment with differentiation-induction agents, normal melanocytes were able to form very few colonies in soft agar, consistent with its requirement for anchorage and spreading for cell growth (Figure III-7). All malignant cells from three different growth phases had more soft agar colonies, compared with normal melanocytes. However, there was no trend detected in the efficiency of soft agar colony formation as a function of growth phase of tumour progression. This finding was incongruous with other cell systems [Herlyn et al, 1985b], which show a direct correlation of soft colony formation with malignant progression. The reason for this discrepancy is not clear, although possible explanations include differences in media requirements, and a minimum cell density requirement at plating.

After exposure to differentiation-induction agents, the effect of differentiation-inducing agents on soft agar colony formation, however, revealed quite distinct findings. Normal melanocytes did not form any colonies, regardless of the induction agent used (Figure III-7). The administration of α-MSH did not cause any alteration in the colony forming efficiency. In contrast, BRDU treatment increased the colony forming efficiency for all cell lines, except WM 1341D, at least five-fold. Most colonies were collections of 8 to 12 cells after cultivation for 14 days. Microscopic examination of the colonies did not reveal significant differences in cell size or shape between the control and BRDU-treated cells. The increased colony formation in soft agar in the presence of BRDU suggest that these induced cells were becoming more anchorage-independent; a phenotype associated with increased malignancy [Bouck and DiMayorca, 1977; van Roy and Mareel, 1992; Nicolson, 1993; Thomas et al, 1993]. Importantly, there was some trend toward different degree of colony forming efficiency based upon the growth phases of tumour progression in the control and BRDU-treated cells. In both conditions, the RGP cells showed the highest colony forming efficiency, followed by VGP cells and MGP cells. The VGP WM 1341D cell line did not show this trend after BRDU treatment, and revealed the least colony forming efficiency, indicating that this cell line may be a more differentiated, less malignant phenotype.

Cell Migration Assay

Before exposure to differentiation-induction agents, the degree of cell migration on Collagen Type I substrate, in response to a soluble Collagen I chemotactic signal was used to determine whether cells from different phases of tumour progression would have different migration rates. All malignant cell lines showed a higher migration rate on this substrate, compared with normal melanocytes (Table III-8). However, no trend was observed in the migration rates between malignant cell lines from different phases of tumour progression. Thus, the higher migration rates on Collagen Type I may be a phenotypic discriminator between normal and malignant cells.

After exposure to differentiation-induction agents, the effect of BRDU on cell migration is given in Table III-6. All cell lines tested, except WM 1341D, had higher migration rates than the control cell lines. Normal melanocytes did not change their rate of migration in the presence of BRDU. The cell line WM 1341D had a decreased migration rate when treated with BRDU. There was no difference in the cell migration rate between the RGP, VGP, and MGP cell lines after exposure to BRDU. The effect of α-MSH was not determined in this assay. Migration rates on Collagen Type I had been suggested to be proportional to metastatic activity [Rusciano and Burger, 1992; van Roy and Mareel, 1992; Thomas et al, 1993; Tsukamoto et al, 1991]. Thus, the increased migration rate of these cells on Collagen Type I in the presence of BRDU may imply that the BRDU-treated cells would become more metastatic *in vivo*. Taken together with the observed changes in cell morphology and colony forming efficiency, BRDU-treated cells exhibit features of a less differentiated phenotype, and could become more metastatic. In contrast, the reduced migration rate observed with WM 1341D corresponded to the more differentiated features observed with BRDU-induction of this cell line. Therefore, migration rate on Collagen I could be a potential phenotypic discriminator for the differentiation phenotype.

Tyrosinase Activity

Before exposure to differentiation-induction agents, the degree of melanization has been indicated to be closely related to the degree of differentiation [Durkacz et al, 1992; Campbell, 1989; Easty et al, 1991; Ruiz-Cabello et al, 1991; Abdel-Malek et al, 1993; Albino, 1992; Yaar and Gilchrest, 1991]. Normal melanocytes have a higher level of dopa-oxidase activity than melanoma cell lines, except SK-MEL 23 (Figure III-9). In fact, with the exception of WM 39 and SK-MEL 23, the melanoma cell lines were amelanotic, and their cell pellet colour corresponded to the degree of tyrosinase activity. The degree of melanization could not discriminate generally between melanoma cells of different growth phases of tumour progression, although it could discriminate between normal and melanoma cells.

After exposure to α-MSH, normal melanocytes did not alter tyrosinase activity whereas several melanoma cell lines increased tyrosinase activity (Figure III-9). RGP WM 35 and VGP WM 39 revealed an 11- and 2-fold increase in tyrosinase activity, respectively, whereas the MGP cell line SK-MEL 23 did not show any increase, although it had a high tyrosinase activity (Figure III-9).

In contrast, BRDU treatment reduced the tyrosinase activity in the RGP, VGP, and MGP cell lines. This was typically seen in the SK-MEL 23 and WM 39 cell lines. Again, normal melanocytes did not alter tyrosinase activity with BRDU treatment. The WM 1341D cell pellet became darker with BRDU-induction, and the tyrosinase activity increased three-fold.

Melanin Content

Melanin content of all cell lines, except normal melanocytes and SK-MEL 23, were low, reflecting the levels of tyrosinase activity (Figure III-10). All cell lines had a light tan to white cell pellet, except normal melanocytes and the MGP SK-MEL 23, which had a dark brown to black cell pellet.

After exposure to BRDU, the VGP WM 1341D cell line produced a darker brown pellet, while the MGP SK-MEL 23 cell line produced a light tan cell pellet. These effects were more prominent than the changes in tyrosinase activity would indicate. Thus, the biological effect of BRDU on melanocytes was more remarkably reflected by melanin content than tyrosinase activity.

BRDU treatment caused a ten-fold increase in melanin content in WM 1341D, and a 40-fold decrease in melanin content in SK-MEL 23. Treatment with α-MSH increased melanin content in WM 35, WM 39 and SK-MEL 23. Thus, changes in melanin content closely mirrored the changes in tyrosinase activity following differentiation induction, suggesting that the observed effects are related to tyrosinase activity.

Effect of BRDU and α-MSH on TRP-1 Expression

Since the RGP and VGP lines of WM 35 and WM 39 were highly responsive to the effects of the two differentiation-inducing agents, the expression of the tyrosinase related protein-1 was examined. Tyrosinase-related protein-1 is a melanosomal glycoprotein associated with melanin pigmentation [Der et al, 1993]. TRP-1 is not expressed by nonpigmented cells and correlates with tyrosinase for melanization [Luo et al, 1994], linking its importance in melanosomal maturation.

After exposure to BRDU treatment, the expression of TRP-1 in WM 39 was decreased, while there was minimally enhanced TRP-1 expression in WM 35-treated cells (Figure III-11). In contrast, α-MSH induced the expression of TRP-1 in both treated melanoma cells.

Those cell lines, which revealed a decrease in tyrosinase activity and melanin content with BRDU treatment, also showed a decrease in the amount of TRP-1 expression. Conversely, treatment with α-MSH increased TRP-1 expression. Thus, the degree of expression of TRP-1 directly correlated with the degree of differentiation (i.e., melanin synthesis and tyrosinase activity).

DISCUSSION

This study has characterized the growth and differentiation properties of human malignant melanocytes in restricted and non-restricted growth phases of tumour progression by employing normal human melanocytes as a control, and by exposing the cells to BRDU and α-MSH. In general, BRDU can inhibit the differentiation of pigment cells causing the decrease or loss of melanin pigmentation whereas α-MSH can stimulate the differentiation of pigment cells with increased melanin pigmentation. However, BRDU is also known to increase the cellular differentiation in several other cell types, including human myeloid leukemia cells [Koeffler et al, 1983], neuroblastoma cells [Feyles et al, 1991], and even some human melanoma cells [Valy-Nagy et al, 1993; Tapscott et al, 1989; Thomas et al, 1993].

Some important observations have been made in this study with respect to the relation between the growth and differentiation properties of normal human melanocytes and their transformed counterparts in RGP, VGP, and MGP of tumour progression. Normal human melanocytes revealed no significant changes in the growth and differentiation properties after exposure to either BRDU or α-MSH. There was no alteration in cell shape, proliferation rate (doubling time), colony forming efficiency, cell migration rate, tryosinase activity, melanin synthesis, degree of melanization within melanosomes and fine structure of melanosomes in maturation processes. In contrast, malignant melanocytes revealed significant responses to both BRDU and α-MSH treatment. Treatment with α-MSH for most cells, except VGP WM 1341D, increased their differentiation state, i.e., tyrosinase activity, melanin synthesis and degree of melanization within the melanosomal compartment, but without causing any significant change in their growth properties, i.e., proliferation rate, colony forming efficiency, and cell migration rate. BRDU, on the other hand, decreased differentiation, but increased the growth properties of all the phenotypic markers. Finally, the effect of these differentiation-inducing agents appeared to be reversible, as the changes in cell growth and differentiation induced by BRDU and α-MSH were reverted to the control state following culture in BRDU or α-MSH free media for 7 days.

Biology of BRDU and MSH

The rationale for using differentiation-inducing agents on various stages of malignant melanoma is to exploit the ability of neural crest cells to change their differentiation phenotype. Neural crest cells have a pluripotential differentiation with a restricted developmental potential [Ito et al, 1993]. Furthermore, this ability to alter differentiation phenotype is controlled by a variety of factors, including soluble and contact factors [Baron, 1993]. Experiments with truncal neural crest cells in quails have demonstrated a plasticity of developmental fate which is dependent on local factors [LeDouarin, 1993]. This includes cells that have already become differentiated. LeDouarin has shown that cells that are supposedly well differentiated are capable of altering their differentiation state to a more primitive state, if the cell is placed in the appropriate environment. She found that the cell can take on the differentiation state appropriate for that location during its migration, implying that these cells still retain a certain level of pluripotency. Normal melanocytes are capable of acquiring a nevoid phenotype, if removed from the epidermis and placed into the dermis [Medrano et al, 1993]. Alternatively, alterations in melanocyte phenotype can occur after exposure to local soluble mediators, such as leukotriene C_4 [Medrano et al, 1993], interferon-τ [Garbe and Krasagakis, 1993], endothelin-1 [Imokawa et al, 1992], or other mesenchymal factors [Campbell, 1989]. The net result would be an alteration in several signal transduction mechanisms, such as G-proteins [Medrano et al, 1993], protein kinase C [Krasagakis et al, 1993], or protein phosphorylation [Halaban et al, 1992].

BRDU is substituted for thymidine in DNA, and it is able to induce many of the biological responses. Theoretically, mispairing during DNA replication, occurs when the enol form of BRDU is incorporated into the genome opposite deoxyguanosine [Freese, 1959]. Alternatively, BRDU can induce a nucleotide base shift from A -> G during DNA strand replication [Szybalski, 1964]. It also increases the frequency of sister chromatid exchanges during the second S-phase and the replication of the BRDU-substituted template [Shiraishi and Sandberg, 1977], leading to increased mutation errors. Furthermore, the substitution leads to a slowed replication rate by DNA polymerase from the G_0 and G_1 phase [Morris et al, 1989], increasing the vulnerability to further substitution. The BRDU-substitution interferes with template RNA formation, increasing the number of errors [Pawloski, 1976].

BRDU is selectively incorporated in specific regions, called fragile sites. A relationship is suggested between the location of chromosomal fragile sites and those regions which replicate their DNA during the later stages of S-phase [LeBeau, 1986]. An association between fragile sites and cancer-specific chromosome rearrangements has been recognized [LeBeau, 1986]. How this selective incorporation regulates the multiple processes necessary for melanosomal maturation is not known, however, these cell lines may be useful in mapping out the genetic control of melanization.

Over the past decade, synthetic α-MSH analogues have been proven to be a powerful tool to study the specific functions of α-MSH on melanocytes and melanoma cells [Sawyer et al, 1980]. [Nle^4-D-Phe^7]-α-MSH (NDP-MSH), a modification of the original 13 amino-acid peptide, was first reported in 1980 [Sawyer et al, 1980], and has been shown to be a very potent MSH agonist. NDP-MSH can prolong melanotropic actions, and protects α-MSH from inactivation by oxidative mechanisms or proteolytic enzymes.

The action of α-MSH is exerted through a membrane-bound receptor [DeLuca et al, 1993; Sawyer et al, 1980; Hunt et al, 1994]. Stimulation of the α-MSH receptor increases both the proliferation and growth in the murine B16 [Lee et al, 1972] or Cloudman S91 melanomas [Wong and Pawelek, 1973]. In pigmented cells, its primary action appears to increase the tyrosinase activity of the cell, without increasing tyrosinase itself [Halaban et al, 1984]. However, in non-pigmented cells, it enhances the production of a pigment-related structural protein, TRP-1, in addition to the increased tyrosinase activity (Halaban et al, 1984]. In murine cells, one effect of α-MSH was the induction of eumelanin synthesis rather than pheomelanin synthesis [Tamate et al, 1981]. Thus, α-MSH is a potent stimulator of melanogenesis in melanoma cells. In certain cell lines, it also affects the cell proliferation and growth. Furthermore, α-MSH can alter murine melanoma cell morphology and increase dendrite formation, particularly when exposed to NDP-MSH [Preston et al, 1987].

Exposure to α-MSH affects the receptor surface expression. However, whether or not the receptor is up- or down-regulated depends on cell type and species. In murine systems, the receptor is down-regulated; whereas the receptor expression is up-regulated in the human melanoma cell lines D10 and 205 [Eberle et al, 1993].

Effect of Tumour Progression on Growth Properties and Differentiation

In the present study, the effect of differentiation induction by BRDU and α-MSH on the growth properties of cells were compared between human cell lines from different growth phases of tumour progression. The cell proliferation rate was not affected by α-MSH treatment, and only the VGP WM 902B was increased by BRDU treatment, indicating that the cell regulatory mechanisms that control cell proliferation were not influenced by either agent. The colony forming efficiency, which reflects the measure of contact-independence, was unaffected by α-MSH treatment, whereas BRDU treatment increased colony formation in all transformed cells in this assay. The RGP WM 35 had the greatest colony formation, followed by the VGP cell lines WM 902B and WM 39, then the MGP cell lines SK-MEL 23 and G 361 (Figure III-7). Finally, cell migration on Collagen Type I, which is an indirect assay of metastatic capacity, was not affected by α-MSH treatment. However, BRDU treatment increased cell migration for all transformed cells, except WM 1341D. No correlation could be detected between cell migration and tumour progression. Taken together, these findings suggest that α-MSH has no detectable effect on the growth properties of human melanocytes of all three phases of tumour progression, while BRDU treatment increases the growth properties of all transformed cells, but does not affect normal melanocytes.

In this study, changes in the process of melanization were used as a measure of cellular differentiation for normal and malignant melanocytes following treatment with BRDU and α-MSH. The dopa oxidase activity of tyrosinase was increased with α-MSH treatment, with the RGP WM 35 being the most sensitive (11-fold elevation), followed by the VGP WM 39 (2-fold increase), while the MGP cell lines SK-MEL 23 and G 361 did not alter their tyrosinase activity. In contrast, BRDU treatment decreased tyrosinase activity in most cells, except VGP WM 1341D, which increased tyrosinase activity 3-fold over control. Unlike the effect observed with α-MSH treatment, the RGP WM 35 cell line was least affected, while the VGP WM 39 cell line (3-fold decrease), and the MGP Sk-MEL 23 cell line (8-fold decrease) were more affected. Hence, an inverse relationship appeared to exist between tyrosinase activity and the stage of tumour progression. The melanin content of cells was also affected by BRDU and α-MSH. Treatment with α-MSH increased melanin content in the RGP and VGP cell lines, but did not affect the melanized MGP SK-MEL 23 cell line. Conversely, BRDU treatment decreased melanin content in the SK-MEL 23 cell line only, with no effect observed in either the RGP or VGP cell lines. The lack of effect of either induction agent on melanization by WM 902B or G 361 is not known, since both cell lines contain tyrosinase. Perhaps a translational defect may exist in these cell lines, since neither cell lines are capable of producing a fully melanized melanosome.

In summary, the effect of α-MSH treatment on cells from different phases of tumour progression does not appear to affect cell growth property, but instead, appears to affect cell differentiation, increasing primarily the degree of tyrosinase activity and TRP-1 expression, wherein the RGP cell lines are the most sensitive, followed by the VGP, and then the MGP cell lines. Melanin content is a less reliable marker than tyrosinase activity for α-MSH action on differentiation. Furthermore, the effect of α-MSH treatment on cell morphology would suggest that most cells acquire a cellular morphology consistent with a more differentiated cellular phenotype, according to Houghton [1987]. In contrast, BRDU treatment decreases tyrosinase activity in an inverse relation to tumour progression. Additionally, the growth properties also increase with BRDU treatment, but in a nonspecific manner. Accordingly, the effect of BRDU treatment on cell morphology revealed a less differentiated cellular phenotype. The opposing effects observed with BRDU and α-MSH treatment on WM 1341D supports these observations, since this cell line became more differentiated with BRDU. It is tempting to consider that the mechanisms responsible for controlling melanization are negatively regulated by cellular mechanisms responsible for controlling cell growth. While most of our findings may support such a relation, the nonspecific increase in growth by BRDU treatment is not in agreement with this position. Nonetheless, human melanomas still remain an excellent model for studying the progression of tumours.

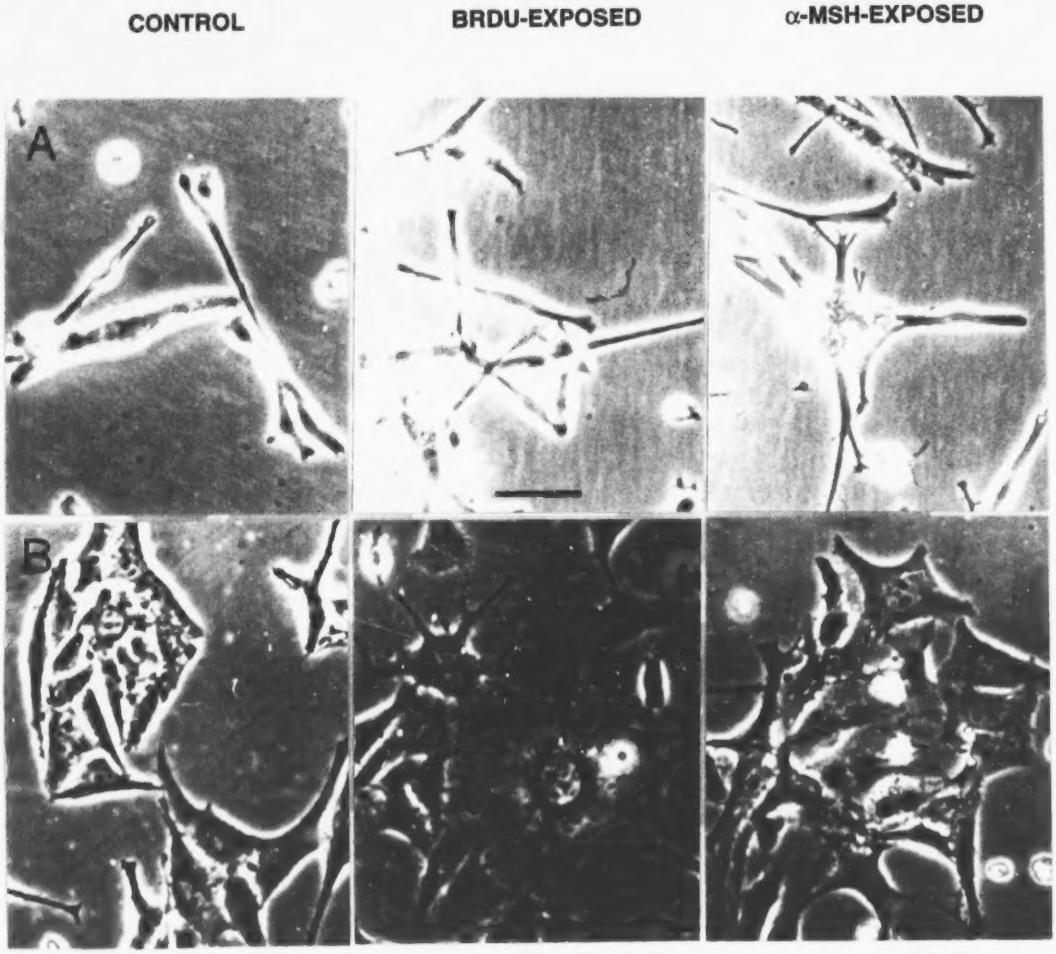

CONTROL
BRDU-EXPOSED
α-MSH-EXPOSED
A
B

FIGURE III-1: Effect of BRDU and α-MSH on Cell Morphology of Normal and Malignant Cells by Light Microscopy.

(a) Normal Melanocytes
(b) Radial Growth Phase Melanoma, WM 35.
See text (above) for details. Bar represents 25 μm.

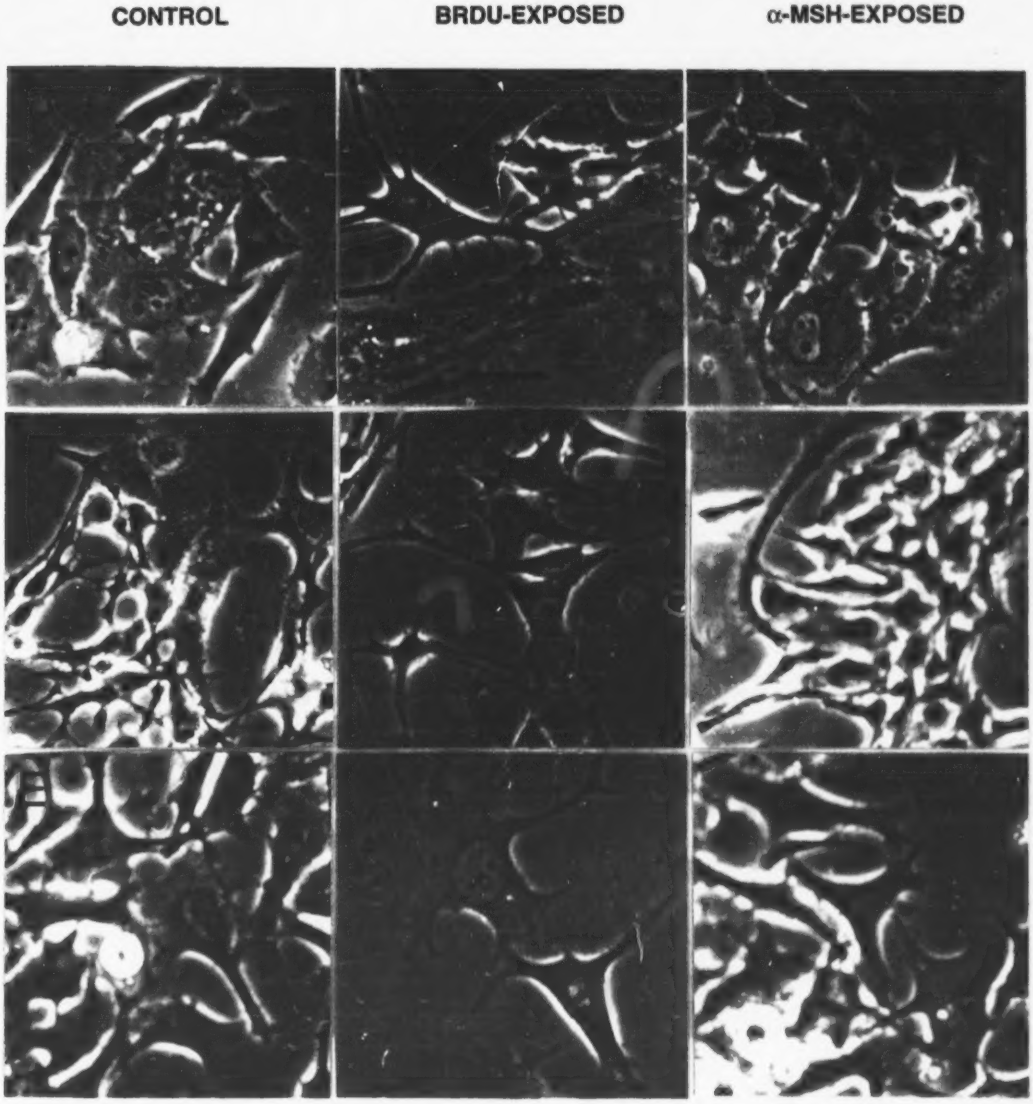

CONTROL
BRDU-EXPOSED
α-MSH-EXPOSED

FIGURE III-1: Effect of BRDU and α-MSH on Cell Morphology of Normal and Malignant Cells by Light Microscopy.

(c) Vertical Growth Phase Melanoma, WM 1341D
(d) Vertical Growth Phase Melanoma, WM 902B
(e) Vertical Growth Phase Melanoma, WM 39
See text (above) for details. Bar represents 25 μm.

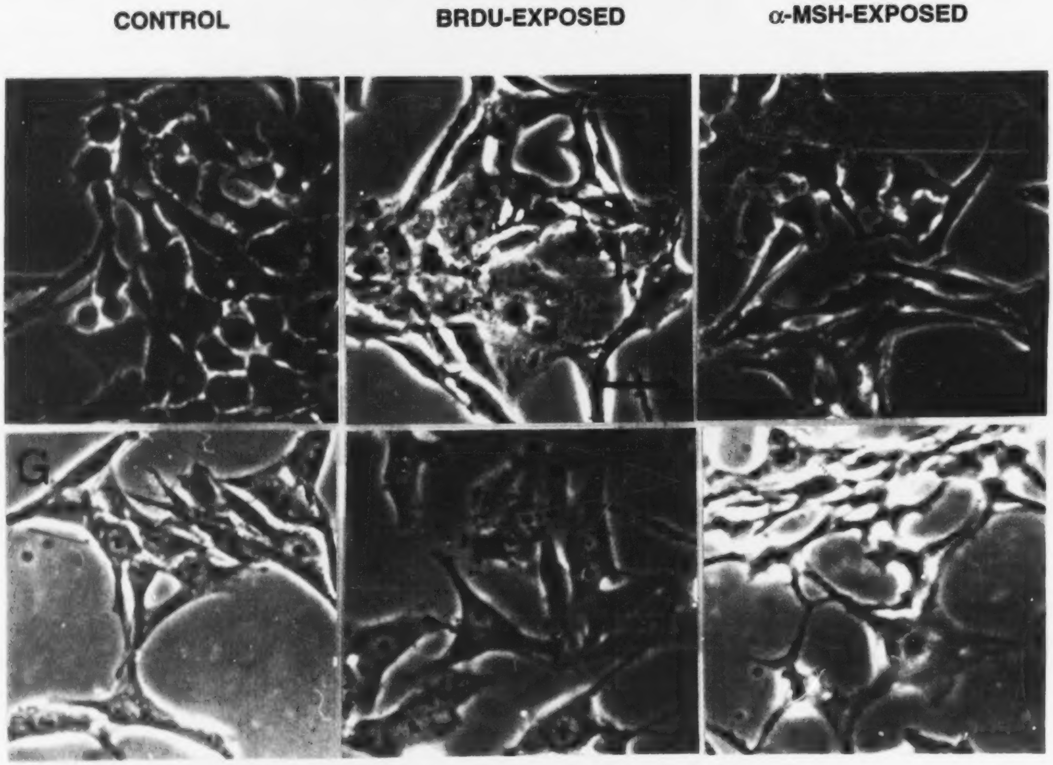

CONTROL BRDU-EXPOSED α-MSH-EXPOSED

FIGURE III-1: Effect of BRDU and α-MSH on Cell Morphology of Normal and Malignant Cells by Light Microscopy.

(f) Metastatic Growth Phase Melanoma, SK-MEL 23
(g) Metastatic Growth Phase Melanoma, G 361
See text (above) for details. Bar represents 25 μm.

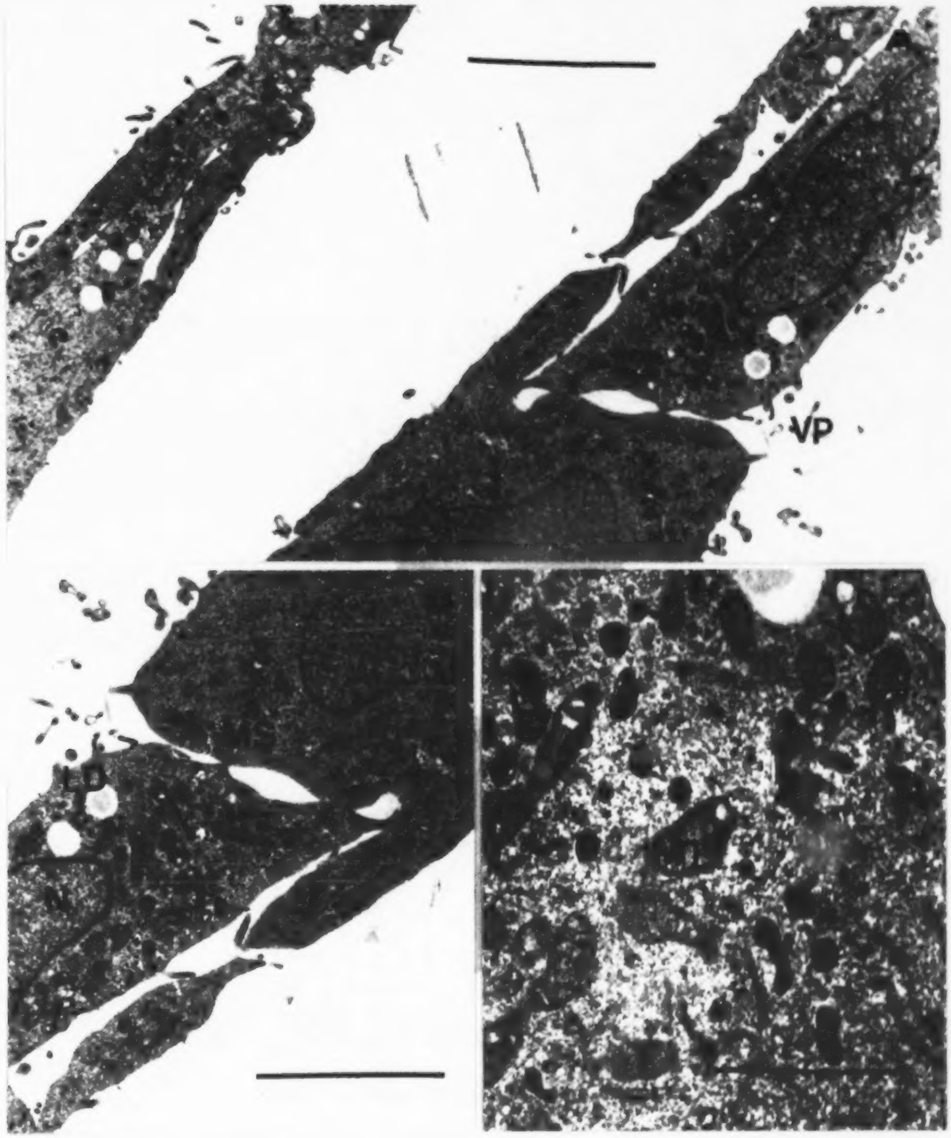


FIGURE III-2: Effect of BRDU and α-MSH on Melanosomal Morphology of WM 35 melanoma cells.

(a) Low power electron micrograph of unexposed WM 35 melanoma cells after 5 days growth showing a fibroblastic morphology with numerous villous projections (VP). Bar represents 1 µm.
(b) High power electron micrograph of unexposed WM 35 melanoma cells showing scattered melanosomes (MS) in various stages of maturation, most of which are Stage III (arrow). N = Nucleus, LD = lipid droplet. Bar represents 1 µm.
(c) High power electron micrograph of an individual melanosome. It is ellipsoidal in shape, individually distributed in the cytoplasm. MT = mitrochondrium. Bar represents 0.5 µm.

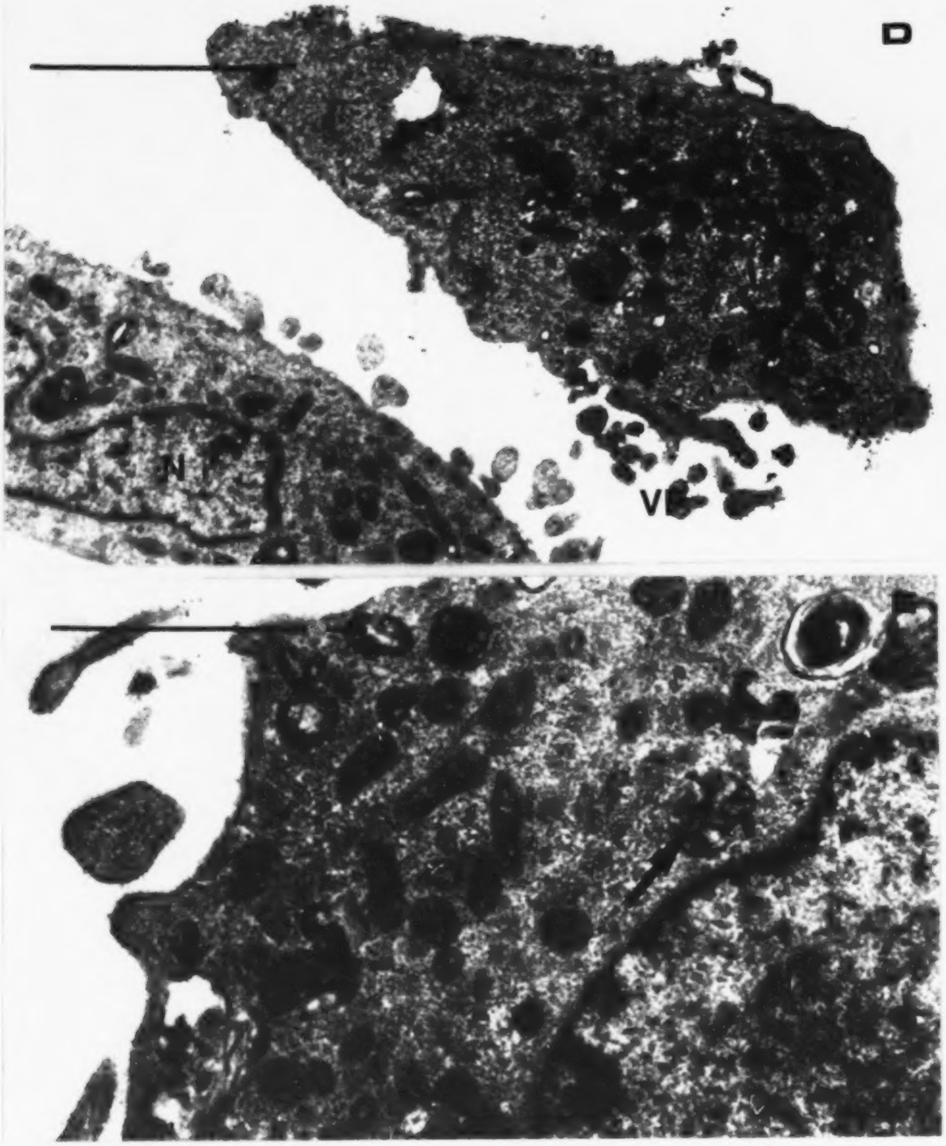


FIGURE III-2: Effect of BRDU and α-MSH on Melanosomal Morphology of WM 35 melanoma cells.

(d) Low power electron micrograph of BRDU-exposed WM 35 melanoma cells, showing no change in the fibroblastic morphology, nor in the degree of melanization compared to unexposed cells (See Figure III-2b,c). N = Nucleus; VP = Villous processes. Bar represents 1.0 μm.

(e) High power electron micrograph of BRDU-exposed WM 35 melanoma cells. There is melanosomal degradation (short arrow) and portions of degraded melanosomes in the cytoplasm (long arrow). The degree of maturation of melanosomes is less than unexposed cells (See Figure III-2c). Bar represents 0.5 μm.

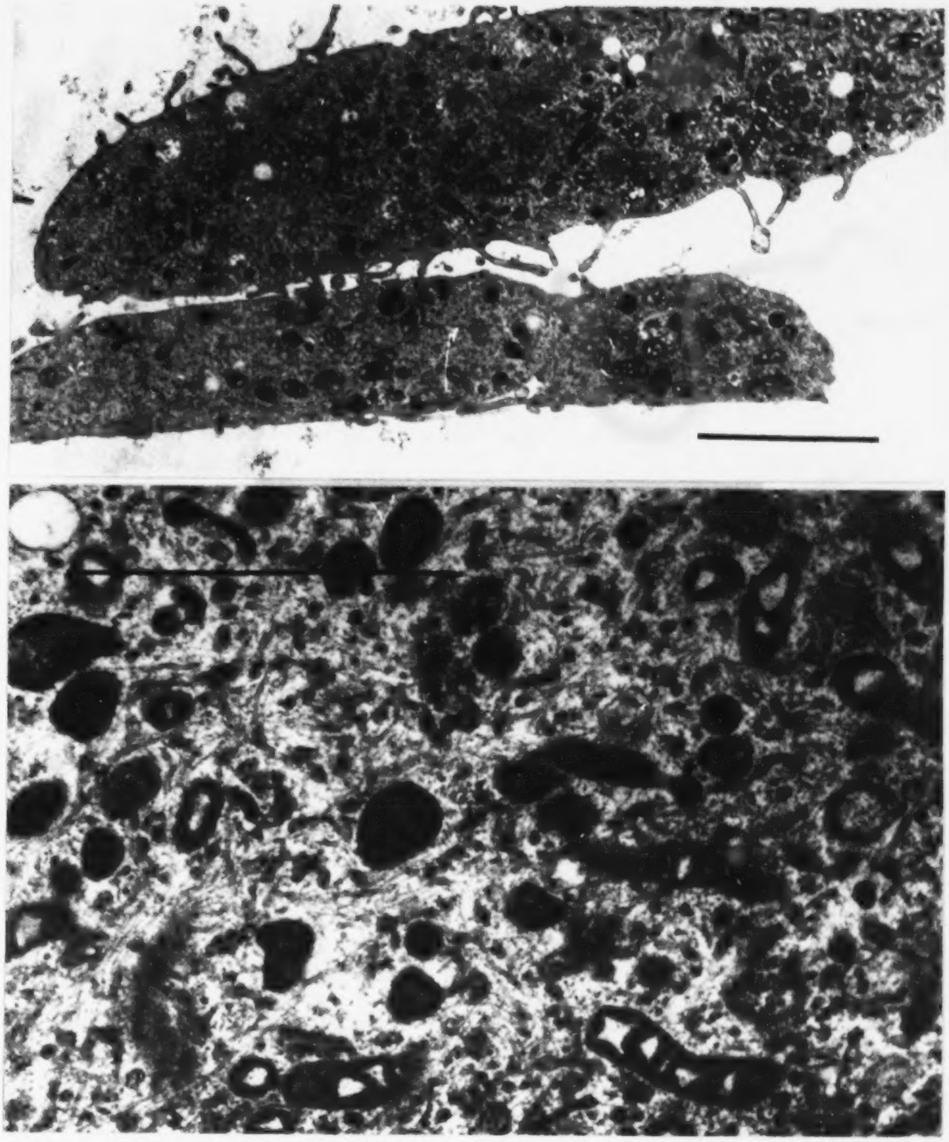


FIGURE III-2: Effect of BRDU and α-MSH on Melanosomal Morphology of WM 35 melanoma cells.

(f) Low power electron micrograph of α-MSH-exposed WM 35 melanoma cells. The cells retain their fibroblastic morphology, and there is a great increase in the number and density of melanosomes, compared to unexposed cells (see Figure III-2b). Bar represents 1.0 μm.
(g) High power electron micrograph of α-MSH-exposed WM 35 melanoma cells showing multiple ellipsoidal, individually dispersed melanosomes of increased maturation, compared to unexposed cells (See Figure III-2c). Bar represents 0.5 μm.

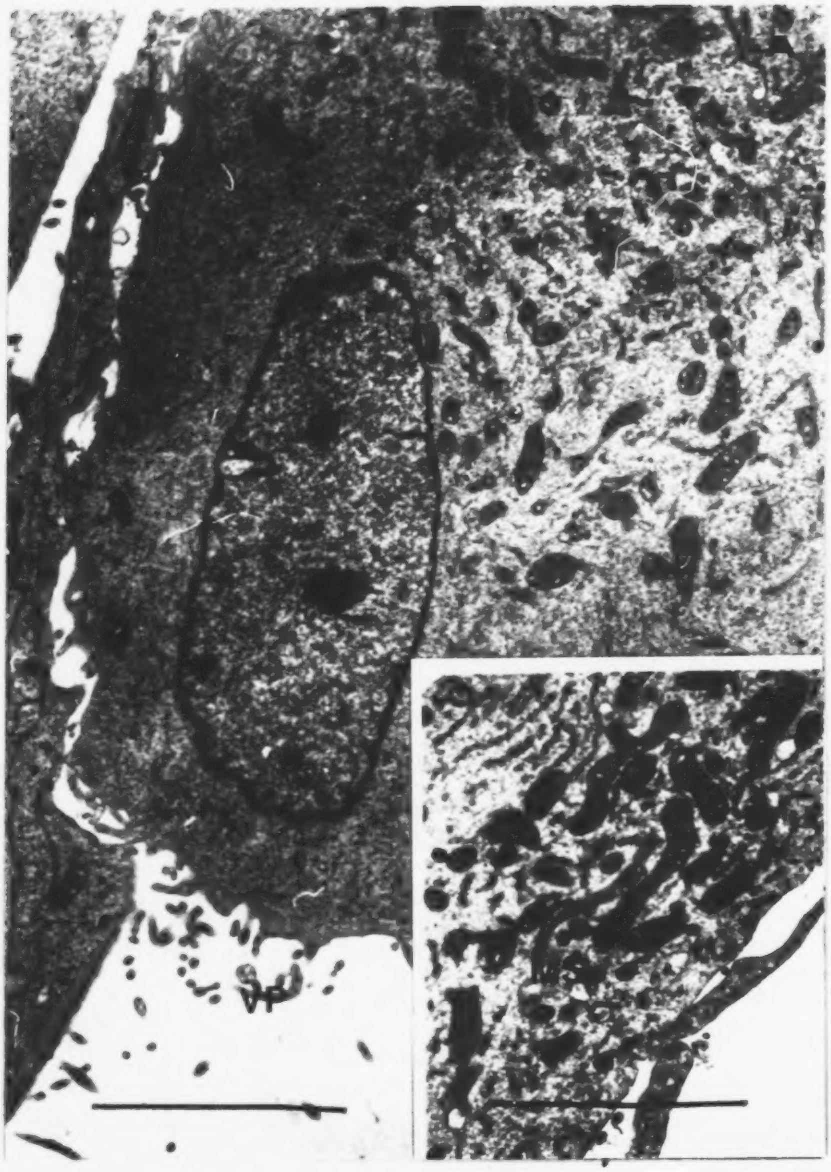


FIGURE III-3: Effect of BRDU and α-MSH on Melanosomal Morphology of WM 39 melanoma cells.

(a) Low power electron micrograph of unexposed WM 39 melanoma cells, showing a fibroblastic morphology. VP = villous processes.
(b) High power electon micrograph of unexposed WM 39 melanoma cells showing a few, individually dispersed, ellipsoidal melanosomes (MS). No focal degradation was observed. Bar represents 1.0 μm.

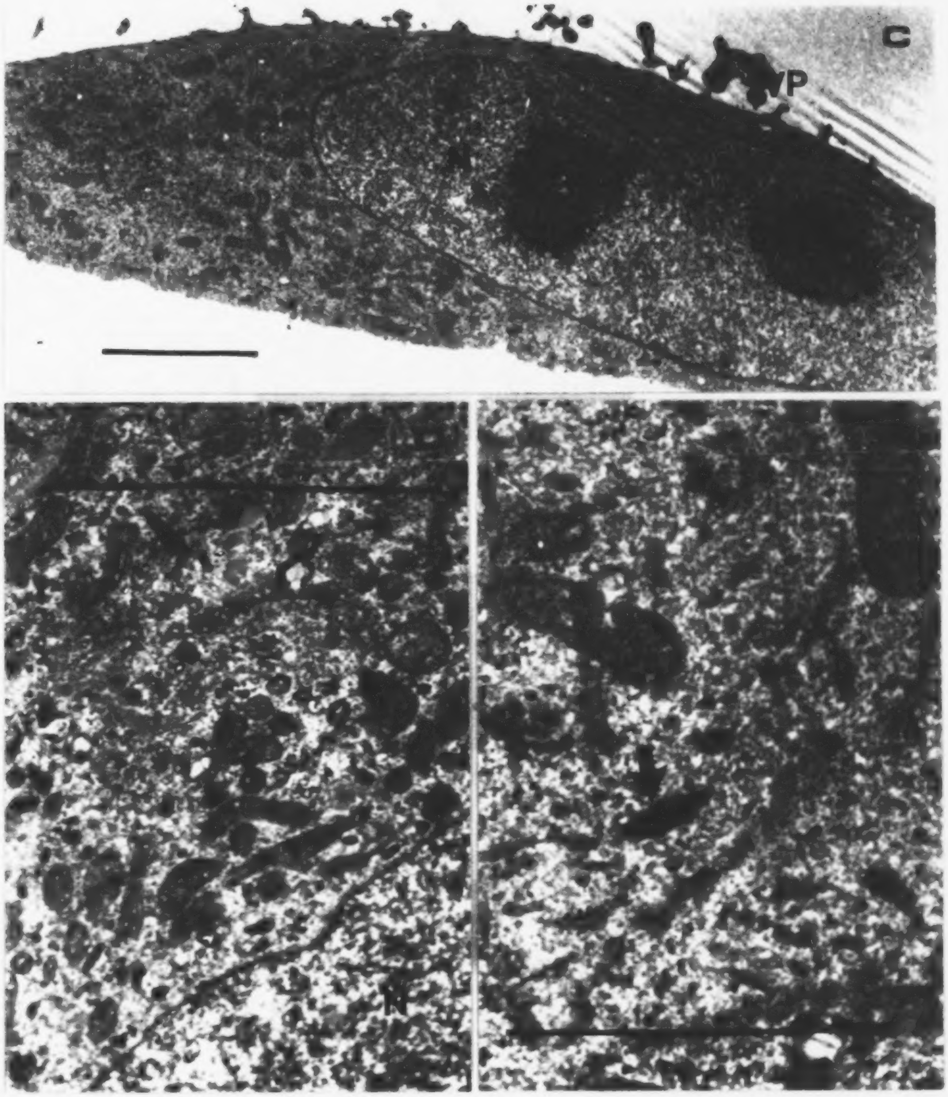


FIGURE III-3: Effect of BRDU and α-MSH on Melanosomal Morphology of WM 39 melanoma cells.

(c) Low power electron micrograph of BRDU-exposed WM 39 melanoma cells showing the fibroblastic morphology. VP = villous processes. N = Nucleus. (d) High power electron micrograph of BRDU-exposed WM 39 melanoma cells showing ellipsoidal, Stage III melanosomes, which are fewer in number compared to unexposed cells (See Figure III-3a). Bar represents 1.0 μm.

(e) High power electron micrograph of WM 39 melanoma cells showing the ellipsoidal Stage III melanosomes (arrow). Bar represents 0.5 μm.

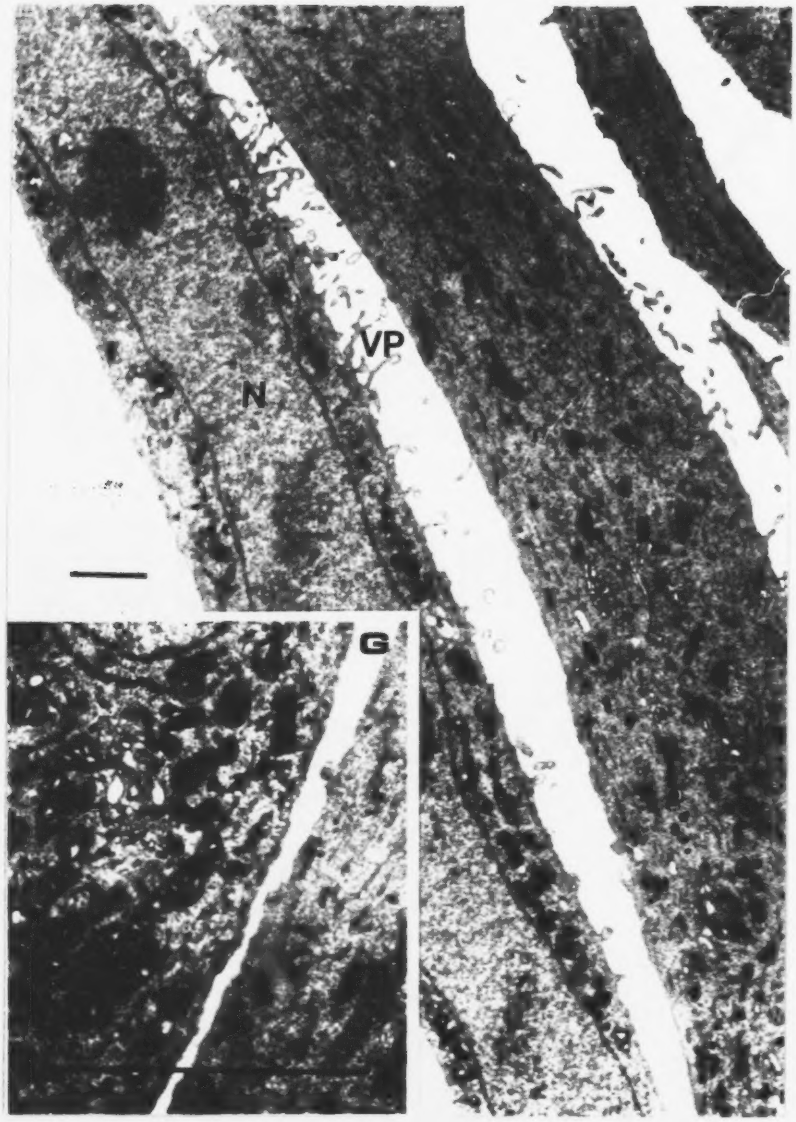


FIGURE III-3: Effect of BRDU and α-MSH on Melanosomal Morphology of WM 39 melanoma cells.

(f) Low power electron micrograph of α-MSH-exposed WM 39 melanoma cells showing the fibroblastic morphology. There are an increased number of melanosomes, compared to unexposed cells (See Figure III-3a). Bar represents 1.0 µm.
(g) High power micrograph showing the degree of melanization is greater than unexposed cells (See Figure III-3b). VP = villous processes. N = Nucleus. Bar represents 1.0 µm.

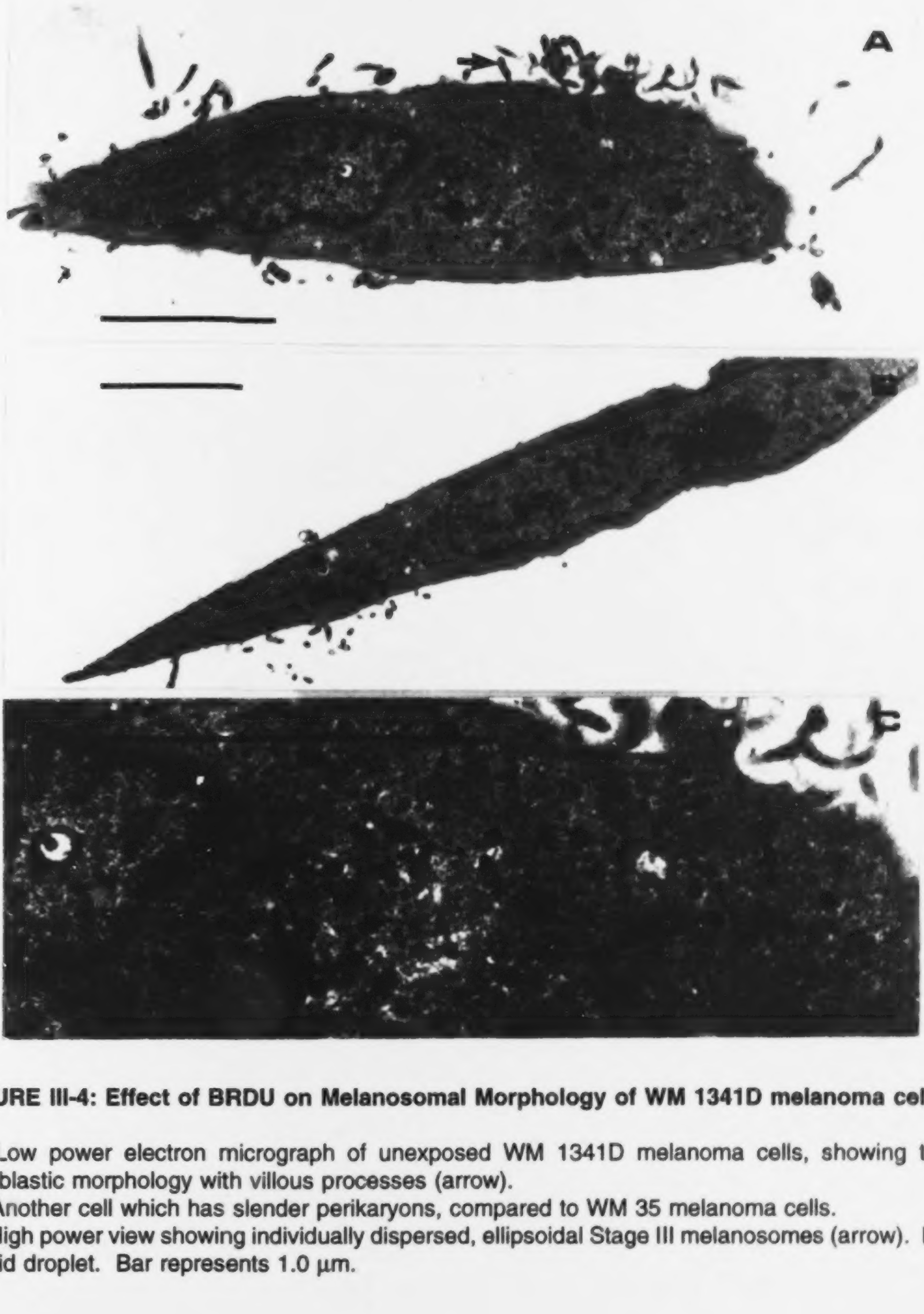


FIGURE III-4: Effect of BRDU on Melanosomal Morphology of WM 1341D melanoma cells.

(a) Low power electron micrograph of unexposed WM 1341D melanoma cells, showing the fibroblastic morphology with villous processes (arrow).
(b) Another cell which has slender perikaryons, compared to WM 35 melanoma cells.
(c) High power view showing individually dispersed, ellipsoidal Stage III melanosomes (arrow). LD = lipid droplet. Bar represents 1.0 µm.

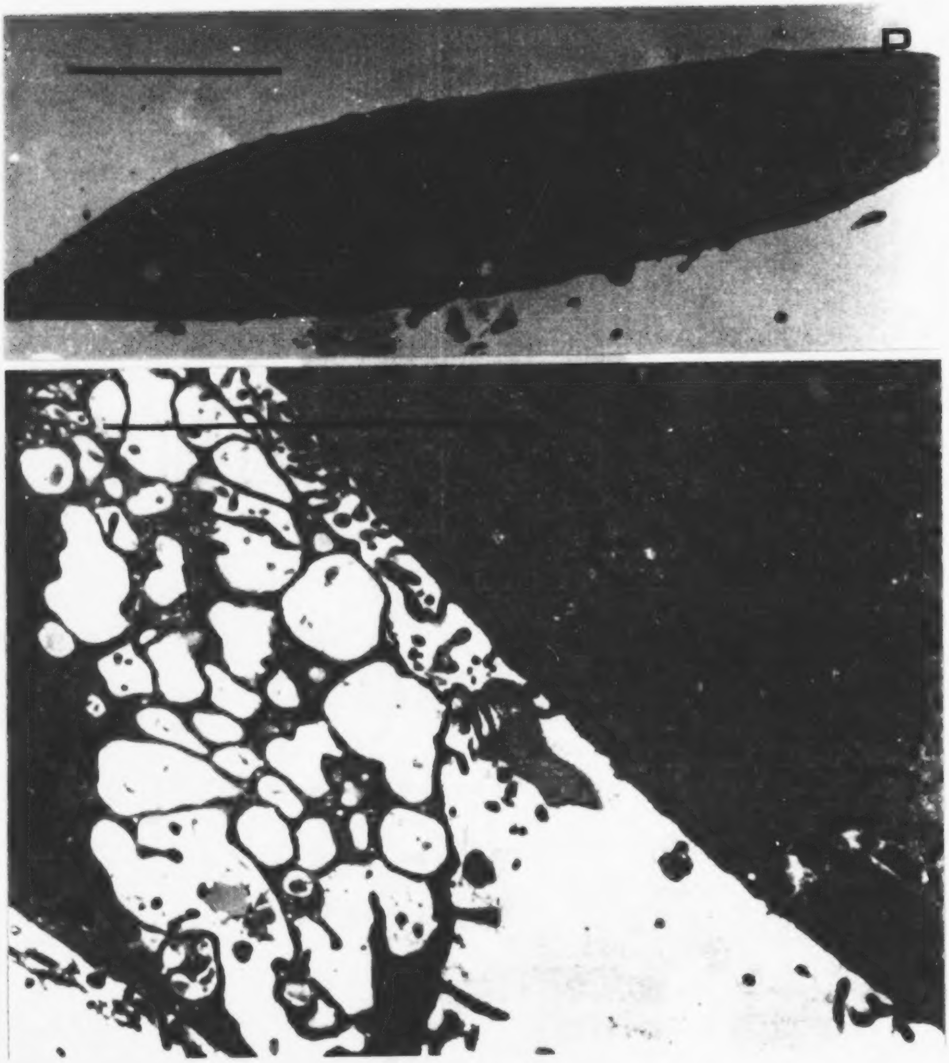


FIGURE III-4: Effect of BRDU on Melanosomal Morphology of WM 1341D melanoma cells.

(d) Low power electron micrograph of BRDU-exposed WM 1341D melanoma cells, with its fibroblastic morphology, and villous processes. There are more electron dense organelles in the cytoplasm, compared to unexposed cells (See Figure III-4a).
(e) High power view, with a high density of individually scattered ellipsoidal Stage IV melanosomes (arrow). Bar represents 1 µm.

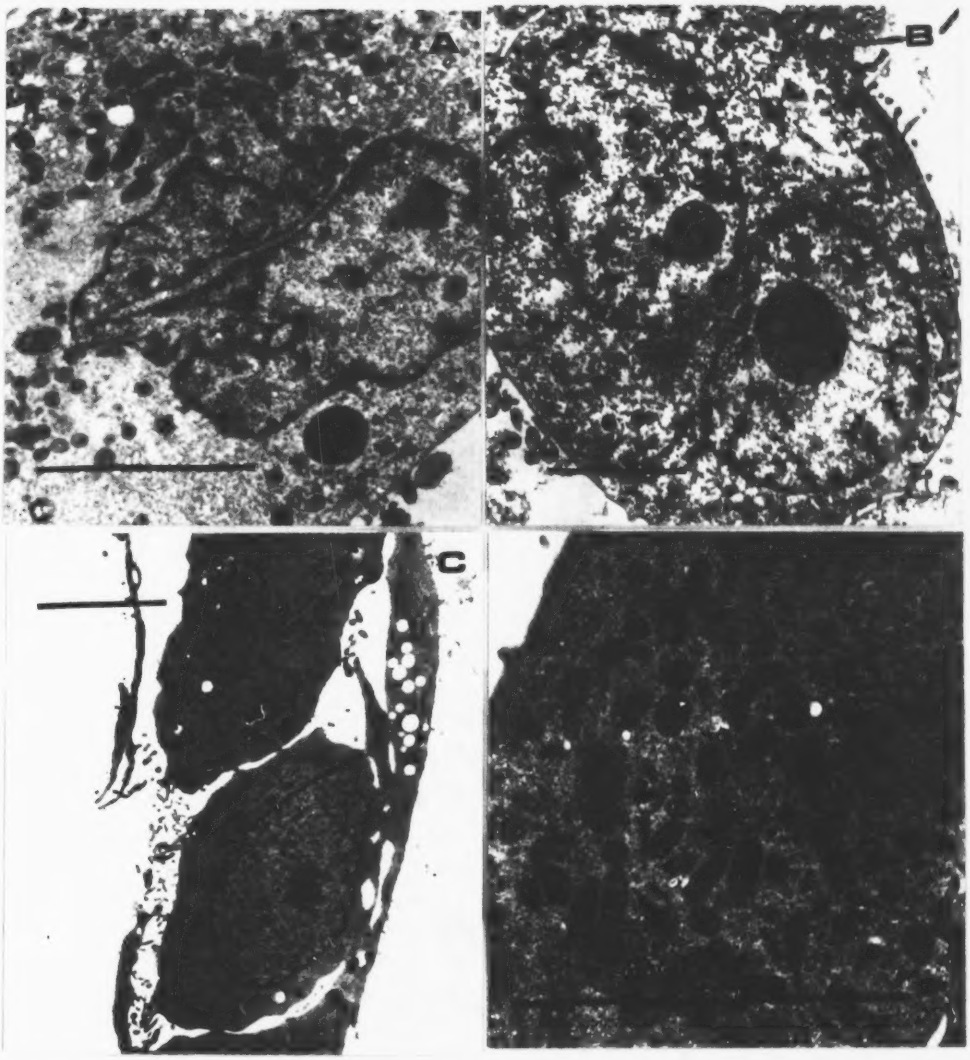


FIGURE III-5: Effect of BRDU and α-MSH on Melanosomal Morphology of SK-MEL 23 melanoma cells.

(a) Low power electron micrograph of unexposed SK-MEL 23 melanoma cells, showing the epithelioid morphology, and moderate density of melanosomes.
(b) Low power view of BRDU-exposed SK-MEL 23 melanoma cells, also showing the epithelioid morphology, but a lack of mature melanosomes.
(c) Low power view of α-MSH-exposed SK-MEL 23 melanoma cells
(d) High power view of α-MSH-exposed SK-MEL 23 melanoma cells showing individual Stage IV melanosomes. Bar represents 1 μm.

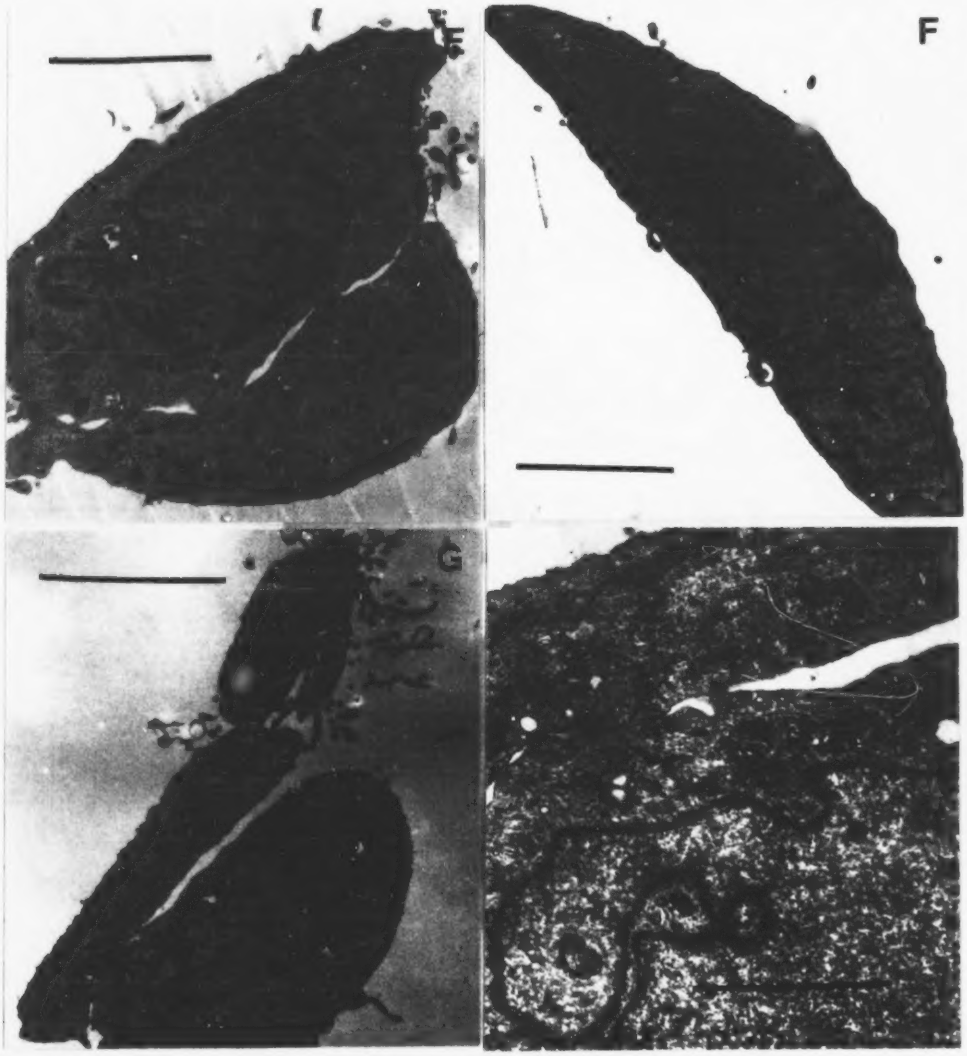
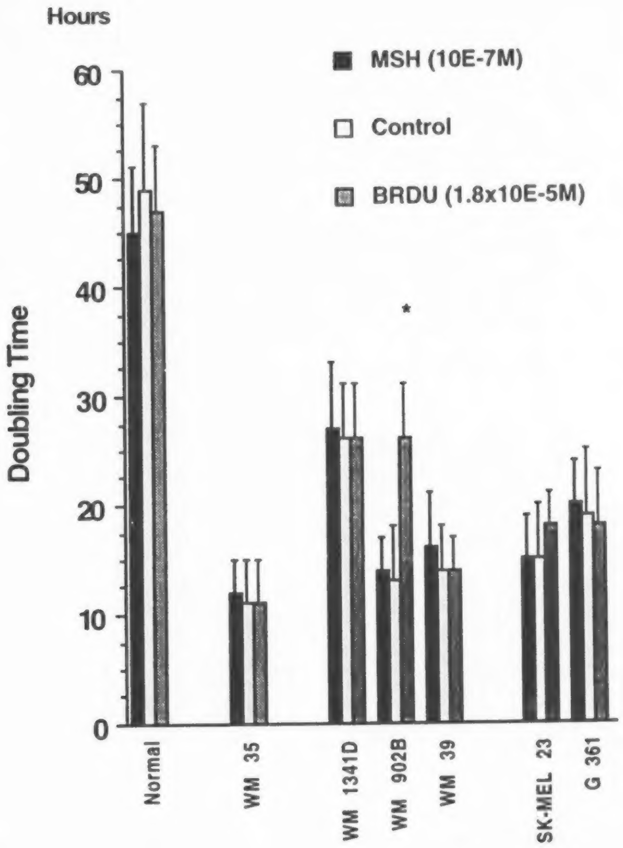


FIGURE III-5: Effect of BRDU and α-MSH on Melanosomal Morphology of G 361 melanoma cells.

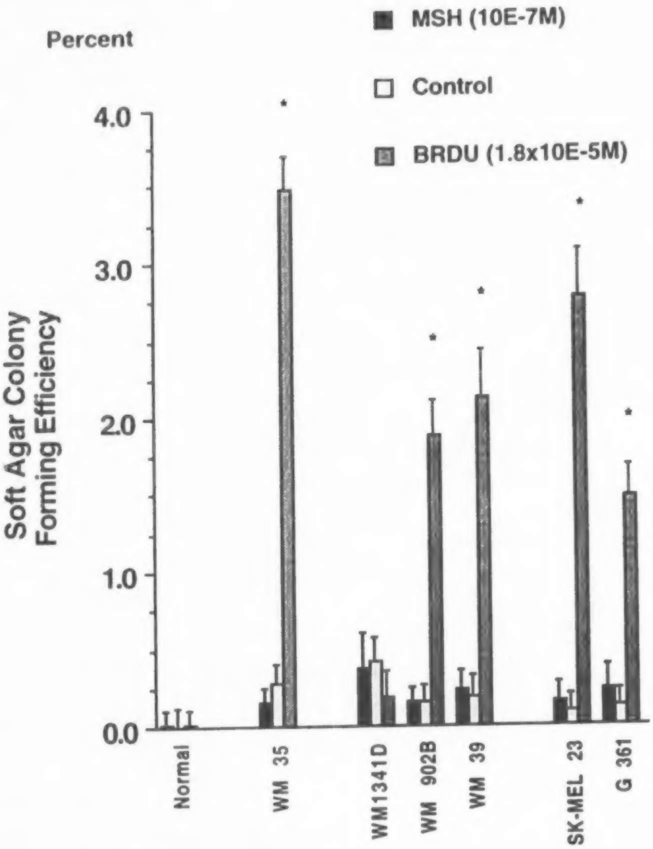
(e) Low power electron micrograph of unexposed G 361 melanoma cells, showing an unmelanized, epithelioid morphology. Bar represents 1.0 μm.
(f) Low power view of BRDU-exposed G 361 melanoma cells, which show no change in cellular and subcellular morphology, compared to the unexposed cells. Bar represents 1.0 μm.
(g) Low power view of α-MSH-exposed G 361 melanoma cells. Bar represents 1.0 μm.
(h) High power view of α-MSH-exposed G 361 melanoma cells, which also show no change in subcellular morphology, compared to the unexposed cells. Bar represents 0.5 μm.



* $p < 0.05$

FIGURE III-6: Effect of BRDU and α-MSH on Doubling Times of Normal and Malignant Melanocytes.

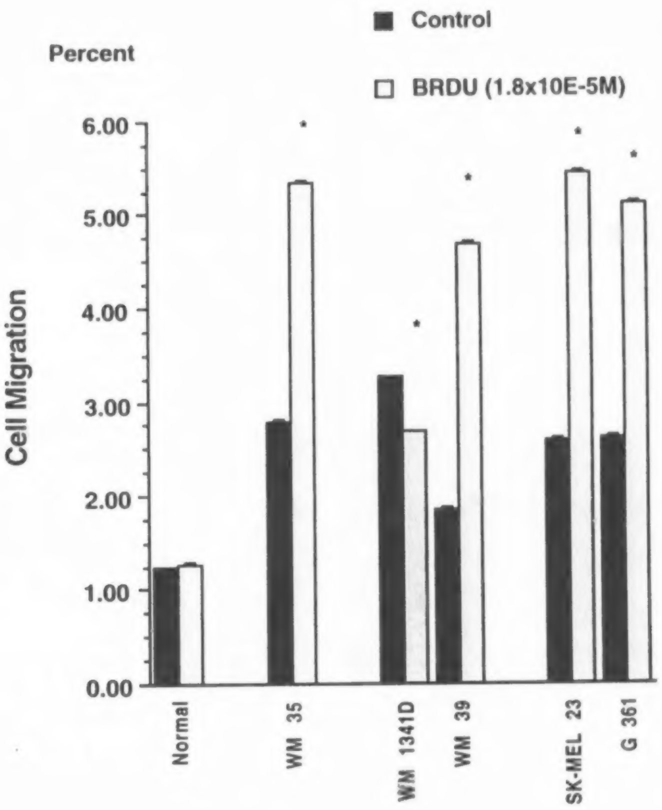
Normal melanocytes had significantly longer doubling times than any melanoma cell line. BRDU exposure of WM 902B cells significantly increased doubling time (asterisk), but not any other cells.



* $p < 0.05$

FIGURE III-7: Effect of BRDU and α-MSH on Soft Agar Colony Formation of Normal and Malignant Melanocytes.

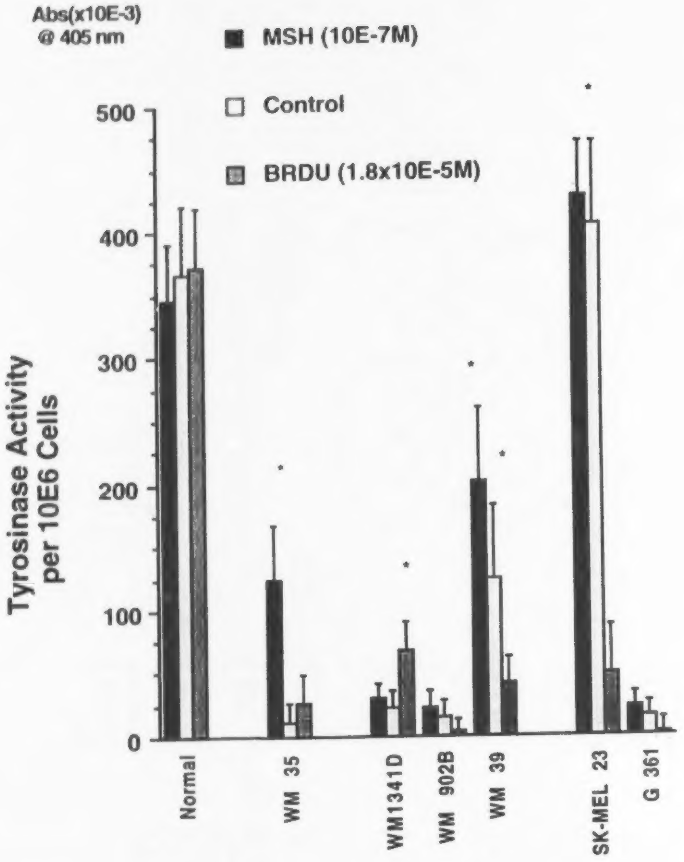
BRDU exposure significantly increased the colony forming ability of all of the malignant cells (asterisk), whereas α-MSH had no effect.



* $p < 0.05$

FIGURE III-8: Effect of BRDU on Cell Migration over Collagen Type I substrate of Normal and Malignant Melanocytes.

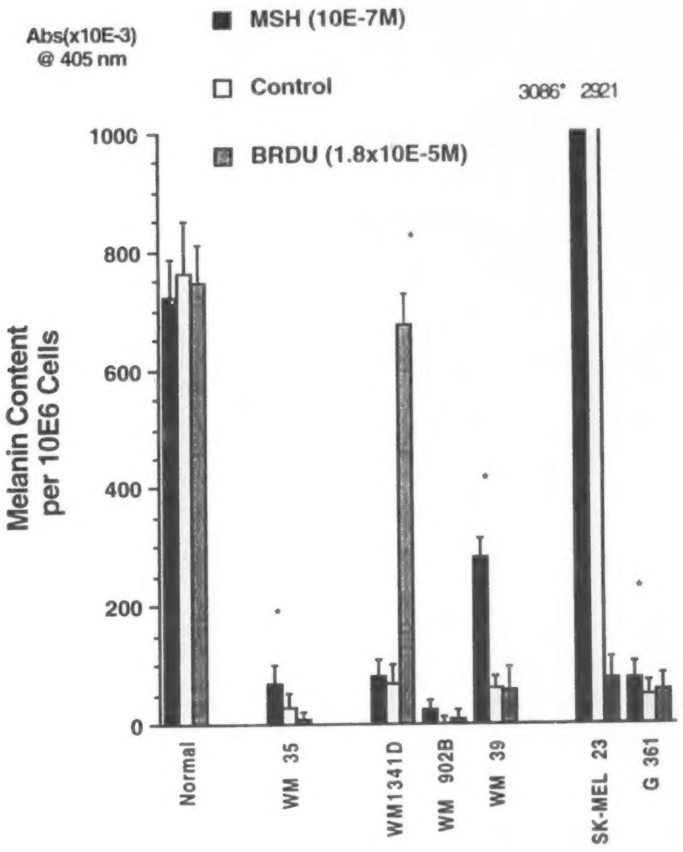
BRDU exposure increased the percent migration of cells through a membrane coated with 10 μg/ml Collagen type I solution, except for WM 1341D, which decreased the migration rate (asterisk).



* $p < 0.05$

FIGURE III-9: Effect of BRDU and α-MSH on Tyrosinase Activity of Normal and Malignant Melanocytes.

BRDU exposure significantly decreased dopa-oxidase activity of cellular tyrosinase (asterisk) in most malignant cells, except for WM 1341D, which increased dopa-oxidase activity. α-MSH exposure increased dopa-oxidase activity in WM 35 and WM 39 cells.



* $p < 0.05$

FIGURE III-10: Effect of BRDU and α-MSH on Melanin Content of Normal and Malignant Melanocytes.

BRDU exposure increased melanin content in WM 1341D cells, and decreased melanin content in SK-MEL 23 cells (asterisk). α-MSH exposure increased melanin content in both WM 35 and WM 39 cells.

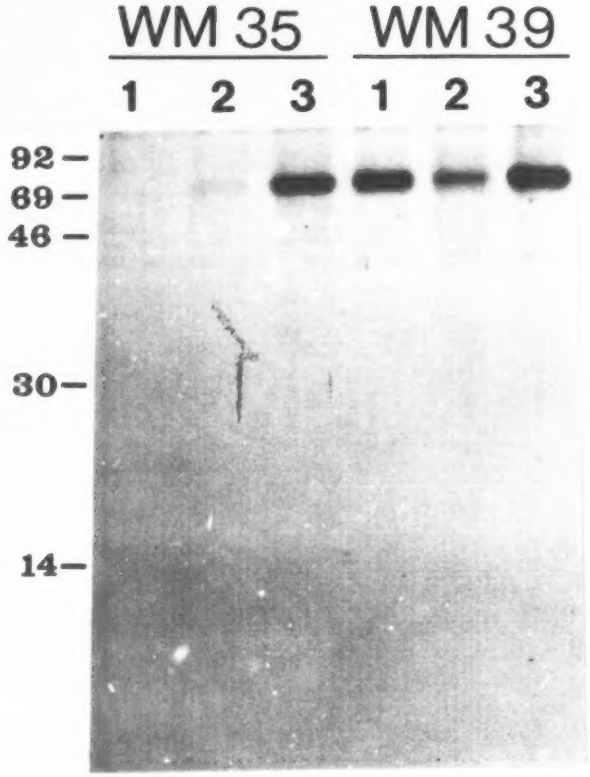


FIGURE III-11: Effect of BRDU and α-MSH on the Expression of Tyrosinase Related Protein-1 of WM 35 and WM 39 Melanoma Cells.

BRDU exposure slightly increased TRP-1 expression in WM 35 cells, but decreased TRP-1 expression in WM 39 cells. Conversely, α-MSH exposure increased TRP-1 expression in both cell lines.
(1) = unexposed, (2) = BRDU exposed, (3) = α-MSH exposed.

Measured Optical Densities: WM 35: (1) 0.00, (2) 0.07, (3) 0.48; WM 39: (1) 0.44, (2) 0.26, (3) 0.51.

REFERENCES

Abdel-Malek Z, V Swope, C Collins, R Boissy, H Zhao, J Nordlund. Contribution of melanogenic proteins to the heterogenous pigmentation of human melanocytes. J Cell Sci. 106:1323-1331, 1993.

Albino AP. The role of oncogenes and growth factors in progressive melanoma-genesis. Pigment Cell Res. Suppl 2:199-218, 1992.

Aso M, K Hashimoto, H Eto, T Fukaya, M Ishihara, S Shimao, Y Shimizu. Expression of Schwann-cell characteristics in pigmented nevus. Cancer 62: 938-943, 1988.

Aubert C, F Rouge, J-R Galindo. Differentiation and tumorigenicity of human malignant melanocytes in relation to their culture conditions. J. Natl. Cancer Instit. 72:3-12, 1984.

Baron MH. Reversibility of the differentiated state in somatic cells. Curr. Opin. Cell Biol 5:1050-1056, 1993.

Bouck N, G Di Mayorca. Evaluation of chemical carcinogenicity by *in vitro* neoplastic transformation. Methods in Enzymology 58:296-302,1979.

Clark WH, DE Elder, D Guerry, MN Epstein IV, MH Greene, M Van Horn. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. Hum. Pathol. 15:1147-1165, 1984.

Campbell S. Melanogenesis of avian neural crest cells *in vitro* is influenced by external cues in the periorbital mesenchyme. Development 106:717-26, 1989.

Delcroix M, A Libert, F Lejeune. Identification of fibroblastoid cell type in human melanoma cultures. J. Natl. Cancer Instit. 77:1171, 1986.

DeLuca M, W Siegrist, S Bondanza, M Mathor, R Cancedda AN Eberle. α melanocyte stimulating hormone (αMSH) stimulates normal human melanocyte growth by binding to high-affinity receptors. J Cell Si. 105:1079-1084, 1993.

Der JE, WT Dixon, K Jimbow, T Horikoshi. A murine monoclonal antibody, MoAb HMSA-5, against a melanosomal component highly expressed in early stages, and common to normal and neoplastic melanocytes. Br. J. Cancer 67:47-57, 1993.

Durkacz BW, J Lunec, H Grindley, S Griffin, O Horner, A Simm. Murine melanoma cell differentiation and melanogenesis induced by poly(ADP-ribose) polymerase inhibitors. Exp.Cell Res. 202:287-291, 1992.

Easty D, IR Hart, K Patel, C Seymour, M Yacoub, A Domscheit, S Gunter, W Postel, A Gorg, MJ Dunn. Changes in protein expression during melanoma differentiation determined by computer analysis of 2-D gels. Clin.Exp.Metastasis 9:221-230, 1991.

Eberle AN, W Siegrist, C Bagutti, J Chluba-De-Tapia. Receptors for melanocyte-stimulating hormone on melanoma cells. in: The Melanotropic Peptides. Ann. New York Acad. Sci. 680:320-341, 1993.

Eisinger M, Marko O. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc Natl Acad Sci USA 79:2018-2022, 1982.

Feyles V, WT Dixon, LK Sikora, RC McGarry, LM Jerry. Human melanoma-associated antigen expression on human neuroblastoma cells: effects of differentiation inducers. Cancer Immunol. Immunother. 32:261-272, 1991.

Freese E. The specific mutagenic effect of base analogues on phage T4. J. Mol. Biol. 1:87-105, 1959.

Garbe C, K Krasagakis. Effects of interferons and cytokines on melanoma cells. J. Invest. Dermatol. 100 (2 Suppl):239S-244S, 1993.

Guiffre L, M Schreyer, J-P Mach, S Carrel. Cyclic AMP induces differentiation *in vitro* of human melanoma cells. Cancer 61:1132-1141, 1988.

Halaban R, SH Pomerantz, S Marchall, AB Lerner. Tyrosinase activity and abundance in Cloudman melanoma cells. Arch. Biol. Chem. Biophys. 230:383-387, 1984.

Herlyn M, G Balaban, J Bennicelli, D Guerry, R Halaban, D Herlyn, DE Elder, GG Maul, Z Steplewski, PC Nowell, WH Clark, H Koprowski. Primary melanoma cells of the vertical grwoth phase: similarities to metastatic cells. J. Natl. Cancer Instit. 74:283-289, 1985.

Herlyn M, J Thurin, G Balaban, JL Bennicelli, D Herlyn, DE Elder, E Bondi, D Guerry, PC Nowell, WH Clark, H Koprowski. Characteristics of culture human melanocytes isolated from different stages of tumor progression. Cancer Res. 45:5670-5676, 1985.

Hirobe T. Control of melanocyte proliferation and differentiation in the mouse epidermis. Pigment Cell Res. 5:1-11, 1992.

Houghton AN, FX Real, LJ Davis, C Cordon-Cardo, LJ Old. Phenotypic heterogeneity of melanoma: relation to the differentiation program of melanoma cells. J. Exp. Med. 164:812-819, 1987.

Hunt G, C Todd, JE Cresswell, AJ Thody. α-melanocyte stimulating hormone and its analogue Nle^4DPhe^7 α-MSH affect morphology, tyrosinase activity and melanogenesis in cultured human melanocytes. J.Cell Sci. 107:205-211, 1994.

Imokawa G, Y Yada, M Miyagishi. Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. J. Biol. Chem. 267:24675-80, 1992.

Ito K, T Morita, M Sieber-Blum. *In vitro* clonal analysis of mouse neural crest development. Dev. Biol. 157:517-25, 1993.

Koeffler HP, J Yen, J Carlson. The study of human myeloid differentiation using bromodeoxyuridine (BrdU). J. Cell Physiol. 116:111-117, 1983.

Krasagakis K, C Garbe, S Kruger-Krasagakes, CE Orfanos. 12-O-tetradecanoylphorbol-13-acetate not only modulates proliferation rates, but also alters antigen expression and LAK-cell susceptibility of normal human melanocytes *in vitro*. J. Invest. Dermatol. 100:653-9, 1993.

Laemmli EK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970.

LeBeau MM. Chromosomal fragile sites and cancer-specific rearrangements. Blood 67:849-858, 1986.

Le Douarin NM, C Ziller. Plasticity in neural crest cell differentiation. Curr. Opin. Cell Biol. 5:1036-1043, 1993.

Lee TH, MS Lee, MY Lu. Effect of α-MSH on melanogenesis and tyrosinase of B-16 melanoma. Endocrinology. 91:1180-1188, 1972.

Liang P, AB Pardee. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967-970, 1992.

Luo D, H Chen, K Jimbow. Co-transfection of genes encoding human tyrosinase and tyrosinase-related protein-1 prevents melanocyte death and enhances melanin pigmentation and gene expression of Lamp-1. Exp. Cell Res. 213:231-241, 1994.

Medrano EE, JZ Farooqui, RE Boissy, YL Boissy, B Akadiri, JJ Nordlund. Chronic growth stimulation of hyman adult melanocytes by inflammatory mediators in vitro: implications for nevus formation and initial steps in melanocyte oncogenesis. Proc.Natl.Acad.Sci.(USA). 90:1790-4, 1993.

Morris SM, LJ McGarrity, OE Domon, WG Hinson, RL Kodell. Flow cytometric analysis of bromodeoxyuridine-induced inhibition of cell proliferation in the human teratocarcinoma-derived cell line, P3. Environ. Mutagen. 14:104-114, 1989.

Nicolson GL. Cancer progression and growth: relationship of paracirne and autocrine growth mechanisms to organ preference of metastasis. Exp. Cell Res. 204:171-180, 1993.

Pawlowski PJ. Effect of 5-bromodeoxyuridine on the appearance of cytoplasmic polyA-containing RNA. J. Cell. Physiol 89:19-27, 1976.

Preston SF, M Volpi, CM Pearson, RD Berlin. Regulation of cell shape in the Cloudman melanoma cell line. Proc.Natl.Acad.Sci.(USA). 84:5247-5251, 1987.

Rusciano D, MM Burger. Why do cancer cells metastasize into particular organs? BioEssays

14:185-194, 1992.

Sawyer TK, PJ Sanfilippo, VJ Hruby, MH Engel, CB Heward, JB Burnett, ME Hadley. 4-Norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone: a higly potent α-melanotropin with ultralong biological activity. Proc.Natl.Acad.Sci.(USA). 77:5754-5758, 1980.

Shiraishi Y, AA Sandberg. The relationship between sister chromatid exchanges and chromosome aberrations in Bloom's syndrome. Cytogenet. Cell. Genet. 18:13-23, 1997.

Skehan P, R Storeng, D Scudiero, A Monks, J McMahon, D Vistica, JT Warren, H Bokesch, S Kenney, MR Boyd. New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. 82:1107-1112, 1990.

Szybalski W, G Ragni, NK Cohn. Mutagenic response of human somatic cell lines. Symp. Int. Soc. Cell Biol. 3:209-221, 1964.

Tamate HB, T Takeuchi. Induction of the shift in melanin synthesis in lethal yellow (A^y/a) mice *in vitro*. Dev. Genet. 2:349-356, 1981.

Tapscott SJ, AB Lassar, RL David, H Weintraub. 5-bromo-2'-deoxyuridine blocks myogenesis by extinguishing expression of Myo D1. Science. 245:532-536, 1989.

Thomas L, PW Chan, S Chang, C Damsky. 5-bromo-2-deoxyuridine regulates invasiveness and expression of integrins and matrix-degrading proteinases in a differentiated hamster melanoma cell. J. Cell Sci. 105:191-201, 1993.

Tsukamoto K, DM Gersten, LW Law, VJ Hearing. Malignant melanoma: relationship to parameters of differentiation. Melanoma Res. 1:223-230, 1991.

Valyi-Nagy, I, I-M Shih, T Gyorfi, D Greenstein, I Juhasz, DE Elder, M Herlyn. Spontaneous and induced differentiation of human melanoma cells. Int. J. Cancer. 54:159-165, 1993.

van Roy F, M Mareel. Tumor invasion: effects of cell adhesion and motility. Trends Cell Biol. 2:163-169, 1992.

Whittaker JR. Quantitative measurement by microdensitometry of tyrosinase (dopa oxidase) development in whole small ascidian embryos. Histochemistry 71:349-359, 1981.

Wollina U, U Kilian U Henkel, H Schaarschmidt, B Knopf. The initial steps of tumor progression in melanocytic lineage: a histochemical approach. Anticancer Res. 11:1405-1414, 1991.

Wong G, J Pawelek. Control of phenotypic expression of cultured melanoma cells by melanocyte stimulating hormone. Nature (New Biol.) 241:213-215, 1973.

Yaar M, BA Gilchrest. Human melanocyte growth and differentiation: a decade of new data. J.Invest.Dermatol. 97:611-617, 1991.

Zeigler-Heitbrock HWL, R Munker, J Johnson, I Petersmann, C Schmoeckel, G Riethmuller. In vitro differentiation of human melanoma cells analyzed with monoclonal antibodies. Cancer Res. 45:1344-1350, 1985.

IV. INTEGRIN EXPRESSION BY NORMAL AND MALIGNANT HUMAN MELANOCYTES DERIVED FROM DIFFERENT STAGES OF TUMOUR PROGRESSION: EFFECTS OF DIFFERENTIATION-INDUCING AGENTS, 5-BROMODEOXYURIDINE AND α-MELANOCYTE STIMULATING HORMONE

INTRODUCTION

The process of tumour growth and metastasis is a cascade of complex events that include the following steps: 1) tumour growth, invasion, and release of neoplastic cells from the primary tumour; 2) movement of tumour cells into the lymphatics or vasculature; 3) survival of the tumour cells with platelets within the circulation and interactions of cells with platelets and with the clotting system; 4) arrest of the tumour cells in distant sites via interactions with vascular or lymphatic endothelium and/or subendothelial basement membrane; 5) migration of tumour cells into tissue parenchyma; 6) Growth of the tumour at the metastatic site [Albelda, 1993]. Many, if not all, of these steps involve either increases or decreases in the ability of tumour cells to adhere to adjacent cells or to surrounding extracellular matrix [Albelda, 1993]. Yet, how these changes in adhesion are accomplished during tumour progression is not understood.

Malignant melanoma has been carefully studied with respect to tumour progression [Clark et al, 1984]. The process of malignant transformation of normal cells has been shown to trigger alterations in integrin expression [Ruoslahti and Giancotti, 1989]. Little information is available about differences between normal melanocytes and malignant melanoma cells in their expression of integrin receptors [Kramer et al, 1991]. Because the development of melanomas takes place in well-defined steps [Clark et al, 1984; 1986] and these cells are easily grown in culture, this system is ideally suited for meaningful comparative studies of integrin expression [Albelda et al, 1990].

This study was performed to firstly, determine whether integrin subunit expression levels by human epidermal melanocytes follow a predictable pattern during tumour progression. The level of integrin expression by normal melanocytes and melanoma cells derived from different representative phases of tumour progression was determined using fluorescence-activated flow cytometry. Specifically, this study is an attempt to determine whether integrin subunit expression would change during the transition from one phase of tumour progression to the next phase. Secondly, the effect of differentiation on integrin expression was examined by measuring the level of integrin subunit expression in cells exposed to the differentiation-inducing agents, BRDU and α-MSH.

This study showed that melanocytes at all stages of tumour progression expressed a limited number of integrin subunits on their surface, and that the expression of the major integrin subunits was greatest in the radial growth phase, with a gradual decrease in expression during tumour progression. Integrin expression was also sensitive to the state of differentiation, determined by the presence of BRDU or α-MSH, and the direction of change of integrin subunit expression reflected the general trend of expression observed with the loss of the differentiation phenotype during tumour progression. Finally, when the potential extracellular matrix ligands were correlated with the observed changes in integrin subunit expression, the tumour phase-specific changes in integrin subunit expression corresponded with the changes in the extracellular matrix components found in the new environment.

MATERIALS AND METHODS

Materials:

Material including cell culture media and supplies were obtained as described earlier (see Chapter III). Monoclonal antibodies against the human α2-integrin [P1E6], α3-integrin [P1B5], α4-integrin [P4G9], α5-integrin [P1D6], αv-integrin [VNR 147], ß2-integrin [P4H9], ß4-integrin [3E1] were generously supplied by Telios (Seattle, WA). Monoclonal antibodies against the ß1-integrin [4B4] subunit was obtained from Coulter (Mississauga, ON). A secondary goat anti-murine antibody conjugated with phycoerythrin was obtained from Becton-Dickinson (Mississauga, ON) and was used for flow cytometry studies (see below).

Cell Lines:

The cell lines and culture conditions were as described in Chapter III (p. 39).

Flow cytometry:

Cells cultivated on plastic substrate were harvested with 10mM EDTA in 0.1 M phosphate buffer (pH 7.4), washed with PBS, and incubated for 30 min on ice with primary antibody at 1:100 dilution in 0.1 M phosphate buffer (pH 7.4). Cells were washed once with PBS for 5 min, and then incubated for 30 min with a secondary antibody conjugated with R-phycoerythrin (Becton-Dickinson, Mississauga, ON) diluted 1:50 in 0.1 M phosphate buffer (pH 7.4). Optimal saturating concentrations were used for both the primary antibody [5 µg/ml purified antibody] and the high-affinity secondary antibody conjugated to a fluorochrome of high quantum efficiency [R-phycoerythrin; log E, 6.0; QY, 0.80]. Cells were washed with PBS for 5 minutes, and fixed with 1% paraformaldehyde in PBS. The fluorescence intensity was measured using a FACScan flow cytometer (Becton-Dickinson, Mississauga, ON), after appropriate gating for the negative control. The median fluorescence intensity and coefficient of variance was determined after counting 10^4 cells. Statistical significance was determined by Student t-distribution, and $p \leq 0.05$ were considered to be statistically significant.

RESULTS

Expression of integrin subunits by flow cytometry on normal and malignant melanocytes

Normal and malignant melanocytes at all phases of tumour progression expressed the ß1-integrin subunit as the predominant ß-integrin subunit at median fluorescence intensity levels significantly greater than fluorescence by secondary antibody alone (Figure IV-1). Malignant cells expressed much higher levels of ß1-integrin than normal melanocytes. The RGP WM 35 cell line had the highest level of expression, followed by the VGP cell lines WM 902B, WM 39, and WM 1341D, then the MGP cell lines SK-MEL 23, and G 361, which had expression levels comparable to the normal melanocytes. Thus, a trend in ß1-integrin expression corresponded to the growth phase of tumour progression, with ß1-integrin expression decreasing with advancing tumour progression.

Normal and malignant melanocytes of all phases of tumour progression did not express ß2-integrin. Expression of ß3-integrin subunit was elevated in RGP WM 35, and VGP WM 39 cells (Figure IV-2), although only WM 35 cells had expression levels approaching statistical significance ($p<0.10$). Therefore, no pattern of ß3-integrin could be observed as a function of tumour progression. Expression of ß4-integrin subunit on the normal melanocytes was not statistically significant from negative control values ($p>0.10$) (Figure IV-3). None of the malignant melanoma cell lines expressed ß4-integrin.

Normal and malignant melanocytes in all phases of tumour progression expressed α2- and α3-integrin subunits as the major α-integrin subunit populations at median fluorescence intensity levels significantly greater than the fluorescence intensity by the secondary antibody alone (Figure IV-4 and IV-5). Expression of α2-integrin subunit was greater for malignant cells than the normal melanocytes. In general, the VGP cell lines (WM 39, WM 902B, WM 1341D) had the highest expression of all growth phases, followed by the RGP cell line WM 35, and finally the MGP cell lines. It appeared that α2-integrin subunit expression increased with the transition to the RGP, was maximal during VGP, and was decreased in the MGP period. This trend was also observed by Albelda [1993], who described an increase in α2 integrin expression on melanoma cells, compared to melanocytes derived from normal skin or melanocytic nevi *in vitro* [Albelda, 1993]. Expression of α3-integrin subunit was greatest in the RGP (WM 35), and progressively decreased during the VGP (WM 39, WM 902B, WM 1341D), and MGP (SK-MEL 23, G 361) to levels slightly above the normal melanocyte expression level (Table IV-1; Figure IV-2).

Expression of α4-integrin subunit was greatest on two of the three VGP melanomas (WM 39 and WM 1341D) at statistically significant levels ($p<0.05$) (Figure IV-6). However, α4-integrin subunit expression was not statistically significant on either normal melanocytes, RGP melanoma cells, WM 35, or the MGP melanoma cells. This trend in α4-integrin subunit expression is consistent with the findings reported by several investigative groups, showing an increased α4-integrin subunit expression by melanoma cells undergoing a transition from radial growth phase to vertical growth phase [Albelda et al, 1990; Cheresh, 1991].

Expression of the α5-integrin subunit on normal and malignant melanocytes was not as great as α2- or α3-integrin subunits (less than 50%), and was not expressed to the same degree on all cell lines (Figure IV-7). Normal melanocytes, WM 35, WM 1341D, and G 361 expressed α5-integrin subunit at a level that approached statistical significance ($p<0.10$), while WM 902B cells expressed α5-integrin subunit at a level that was significantly greater than control fluorescence ($p<0.05$). A trend was suggested in the increased expression of α5-integrin subunit in the VGP melanomas, and a decreased expression by the MGP melanomas, however, the levels of expression were not very significant, and, thus, could be subject to greater variablility.

Expression of αv-integrin subunit was detectable on normal melanocytes, the RGP melanoma and the MGP melanomas (Figure IV-8). However, significant αv-integrin subunit expression by the VGP melanoma cell lines (WM 39, WM 902B and WM 1341D) was not detected. Only the αv-integrin subunit expression by G 361 was at a level that approached statistical significance ($p<0.10$). This is in contrast with the findings of Albelda and associates [1990] and Cheresh [1991], who indicated an increasing level of αvß3 expression by malignant melanoma cells *in vitro* with respect to tumour progression and metastasis *in vivo*. However, the dichotomy between our findings and that of Albelda and associates could be attributed to the use of different cell lines, tumour heterogeneity, and the difficulty with predicting tumour behaviour *in vivo*, based upon observations of cells cultivated from these same tumours *in vitro*.

Since αv-integrin subunit can form an heterodimer with one of several ß-integrin subunits, a comparison was made between the expression of αv-integrin and ß3-integrin subunits, the only ß-integrin subunit for which an antibody was available. One cell line (WM 35) had elevated levels of both αv- and ß3-integrin subunit expression, which might imply that αvß3 was the heterodimer expressed on the cell surface. However, several other cell lines (normal melanocytes, SK-MEL 23 and G 361) had elevated levels of αv-integrin subunit expression, but no ß3-integrin subunit expression. Since only α-ß heterodimers can be expressed on the surface, this data implies that the αv-integrin subunit was coupled with another ß-integrin subunit. Currently, αv-integrin subunit has been suggested to form heterodimers with ß1-, ß3-, ß5-, and ß7-integrin subunits [Hynes, 1992]. Thus, the αv-integrin subunits which were detected on normal melanocytes, SK-MEL 23 and G 361 cells may be coupled to at least one other ß-integrin subunit. This is supported by the finding that normal melanocytes do not express large levels of ß3-integrin subunit, but express ß5-integrin subunits instead [Kramer et al, 1991]. The significance of αv-integrin subunit switching between different ß-integrin subunits is not currently known.

Effect of BRDU and α-MSH on the expression of integrin subunits on normal and malignant melanocytes

Phenotypic heterogeneity has been a concern in observing changes in cellular differentiation, since tumour cell lines are not normally derived from the same host. Differentiation-inducing agents may change the cellular differentiation phenotype of a single cell line, enabling comparisons of cellular phenotypic parameters between control and exposed cells. In order to minimize the effect of phenotypic heterogeneity between cell lines on the variability of integrin expression by cells derived from tumours at different stages of tumour progression *in vivo*, the integrin expression by normal and malignant melanocytes was determined following exposure to BRDU or α-MSH.

According to experiments already described (see Chapter III), the RGP melanoma cell line WM 35, the VGP melanoma cell line WM 39, and the MGP melanoma cell line SK-MEL 23 exhibited a less differentiated cellular phenotype following exposure to BRDU (18 μM) for 7 days. Conversely, the VGP melanoma cell line WM 1341D, exhibited a more differentiated cellular phenotype following BRDU exposure, as did WM 35 and WM 39 following exposure to α-MSH (10^{-7} M) for 7 days. Only α2-, α3-, α5-, and ß1-integrin subunits which are expressed significantly on melanocytes and melanoma cells were examined.

The α3-integrin subunit expression increased during the transition between normal and RGP

(Figure IV-5), and progressively decreased during the transitions between RGP and VGP, and VGP and MGP. Accordingly, RGP WM 35 cells exposed to BRDU became less differentiated, and the α3-integrin subunit expression decreased (Figure IV-9). Conversely, the VGP WM 1341D cell line had a more differentiated phenotype following BRDU exposure, and the α3-integrin subunit expression increased. As seen in Figure IV-9, changes in the differentiation state following exposure to differentiation-induction agents produced significant changes in integrin expression. Furthermore, the direction of change induced by these agents reflected the direction of change observed with transitions between tumour growth phases. Thus, the changes in integrin expression observed as a result of changes in differentiation reflected the changes in tumour progression. These trends were also observed with α2-, α5-, and ß1-integrins (Figure IV-10 to IV-12). Taken together, these findings support the hypothesis that cells undergoing tumour progression also develop a less differentiated phenotype. Also, the patterns of integrin expression observed at each tumour phase is linked with the state of differentiation. Thus, cells at a particular phase of tumour progression acquire a specific differentiation phenotype, and the level of integrin expression is linked in some fashion to this differentiation phenotype.

DISCUSSION

This study has characterized the expression of integrin subunits by melanocytes in restricted and nonrestricted growth phases of tumour progression by using normal melanocytes as a control, and by exposing the cells to the differentiation-induction agents, BRDU or α-MSH. In general, melanocytes and melanoma cells from all phases express a limited number of integrin subunits, and the degree of expression changed with tumour progression. The expression levels of the major integrin subunits (α2, α3, and ß1) increased from levels measured on normal melanocytes to a peak expression level observed with the RGP WM 35 cell line, and decreased during the progression through the VGP and MGP cell lines (Table IV-1). Furthermore, exposure of cells to BRDU or α-MSH caused changes in integrin expression which followed the same trends seen with the loss of differentiation during tumour progression. Thus, the observed changes in integrin expression during transition from one phase to another may reflect important roles for these integrin subunits while the cells traverse that phase of tumour progression.

Tumours exhibit substantial heterogeneity between individual cells *in vivo* [Albino, 1992; Lynch et al, 1991]. Extraction of cells from a tumour *in vivo* and selective cultivation *in vitro* can cause substantial changes in cellular phenotype. It is very possible that the cellular phenotype of an immortal cell line derived from a clinical tumour may be representative of only a small minority of the original cells *in vivo*. Therefore, any comparisons between observations made using cell lines *in vitro*, and the clinical tumour stage *in vivo*, should always be interpreted cautiously.

Foulds (1969) established that the development of malignant tumours follows a stepwise alteration in cell behaviour and morphology. This change in phenotype represents the process of tumour progression [Kramer et al, 1991]. It is likely that a successful phenotype include a suitable array of integrin receptors that would directly determine the adhesive properties of cells and thus influence invasion and metastatic potential [Kramer et al, 1991]. This is because the ability of tumour cells to invade the dermal-epidermal basement membrane and then penetrate the interstitial extracellular matrix is dependent on their capacity to adhere to, interact with, and migrate through these matrices [Kramer et al, 1991].

Changes in $\alpha3\beta1$ expression by melanoma cells in undergoing phenotypic transition may be responsible for the alterations observed in tumours *in vivo*. Immunoelectron microscopy of cultured cells demonstrated that $\alpha3\beta1$ was strongly associated with intercellular contacts [Gailit and Clark, 1993]. Furthermore, epithelial tumours which have rare metastatic activity, such as basal cell carcinoma, have elevated expression of $\alpha3\beta1$ [Ruoslahti and Giancotti, 1989]. Hence, melanoma cells in transition from a more to a less differentiated phenotype may down-regulate surface expression of adhesion molecules responsible for cell-cell and cell-substrate adhesion. This decreased adhesion would release the cell, permitting it to migrate with fewer restrictions.

One of the first events in tumour metastasis is the activation of migratory ability of the malignantly transformed cells. The acquisition of this ability represents a fundamental step in the transition to the invasive mode and there is evidence that integrins may play a regulatory role in motility [Mortarini and Anichini, 1993]. In the very early stages of tumour growth, decreased adhesion to basement membrane or matrix proteins may be advantageous [Albelda, 1993]. The integrity of the basement membrane surrounding tumours is also affected. In many studies examining the composition of the basement membrane in epithelial tumours, a relationship between the structural integrity of the basement membranes and the expression of integrins appeared to exist. Those tumours that were more invasive and lack intact basement membranes tended not to express basement membrane protein binding integrins [Albelda, 1993]. Selection pressures during the metastatic process would favour those cells that have enhanced capacity to attach to and migrate through cellular and extracellular matrix barriers [Kramer et al, 1991]. Furthermore, enhanced expression of integrins on tumour cells after they reach the circulation may aid implantation and promote metastasis [Albelda, 1993]. Many studies have shown that tumour cells which acquire the integrins necessary for invasion and metastasis also tend to have a less differentiated phenotype [Albelda, 1993; Mortarini and Anichini, 1993].

The diverse number of potential integrin adhesion receptors and their promiscuous ligand specificity suggests that there may not necessarily be one specific integrin profile that favours metastasis [Kramer et al, 1991]. Marked heterogeneity is common for some of the α-integrin subunits of the ß1 and ß3 integrin families [Mortarini and Anichini, 1993]. This differential expression correlated with the degree of differentiation [Mortarini and Anichini, 1993]. One study examining five tumour clones derived from a single tumour demonstrated that the clones which expressed multiple ß1 integrins also had the lowest melanin content and expressed an epithelioid morphology [Anichini et al, 1990]. The association between integrins and progression of melanoma does not involve a single receptor, and an increased expression of all receptors is not necessary. Rather, it appears that the whole repertoire of extracellular matrix receptors is subjected to a complex alteration as the neoplastic population evolved to acquire invasive and metastatic capacity [Mortarini and Anichini, 1993].

The present study shows that despite the differences in the cellular differentiation noted between normal and malignant cell lines, no simple patterns were noted in integrin expression. Several integrin subunits with overlapping substrate affinities were expressed on both normal and malignant melanocytes. No single cell line expressed a unique subunit, but the degree of integrin subunit expression between cell types was quite distinct. Neither the αv- or ß3-integrin subunits were highly expressed, nor was a pattern of increasing expression with decreasing differentiation noted. Though this appears contrary to the findings of Albelda et al. [1990], it may be reconciled by the heterogeneity between tumour cell lines.

In summary, the pattern of integrin expression on tumour cells *in situ*, like that in cultured cells, appears to be complex and dependent on the tumour type [Albelda et al, 1990]. The information that integrins may be critical receptors for multiple steps of the metastatic process therefore, has opened the door for research efforts attempting to inhibit metastasis by disrupting the integrin-ligand interaction [Mortarini and Anichini, 1993]. These data may support the hypothesis that interference with integrin function could contribute to the development of treatments to block or prevent the metastatic process [Mortarini and Anichini, 1993]. Because of the heterogeneity observed among tumour cells and between tumours at different growth phases, it is difficult to identify clear differences between the integrin repertoire of melanocytes derived from normal skin compared with those derived from primary or metastatic melanomas, with the possible exception of an increased expression of the α4 subunit on cells derived from malignant tumours [Albelda et al, 1990]. There appears to be a significant difference in the expression of the ß3-integrin subunit by benign melanocytes and melanomas in the radial growth phase (nontumorigenic lesions) versus those melanoma cells in tumourigenic lesions ie, the VGP or metastatic lesions [Albelda et al, 1990]. Clinically, only those melanomas that express VGP and MGP characteristics have the metastasic capacity. Since the distinction between early VGP and RGP by histopathological examination can be difficult, the *in vivo* expression of the ß3-integrin subfamily by melanoma cells with tumourigenic potential could be useful as a marker of cells entering this phase of malignant progession [Albelda et al, 1990].

Table IV-1: **Expression of Integrin Subunits in Normal Melanocytes and Transformed Cells in Restricted and Non-restricted Phases of Tumour Progression**

		Tissue Compartment (Major ECM Component)		
		Epidermis (CIV, LN, Epiligrin)	Dermis (FN, CI)	Distant Organs (FN, LN)
Integrin Subunit	Putative ECM Ligands	N→RGP	RGP→VGP	VGP→MGP
α2	COLL, LN	↑	↑	↓
α3	LN, Epiligrin, COLL	↑	↓	↓
α4	FN, VCAM-1	↔	↑↑	↓
α5	FN	↔	↑	↓
αv	FN, Fg	↔	↑	↑↑
β1	Multiple	↑	↓	↓
β3	FN, Fg	↑	↓	↓
β4	LN	↓	↔	↔

ECM = extracellular matrix
FN = fibronectin
LN = laminin
COLL = collagen
CIV = collagen type IV
CI = collagen type I
Multiple = FN, LN, CI, CIV, fibrinogen, vitronectin
↓ = decreased expression
↑ = increased expression
↔ = no change in expression

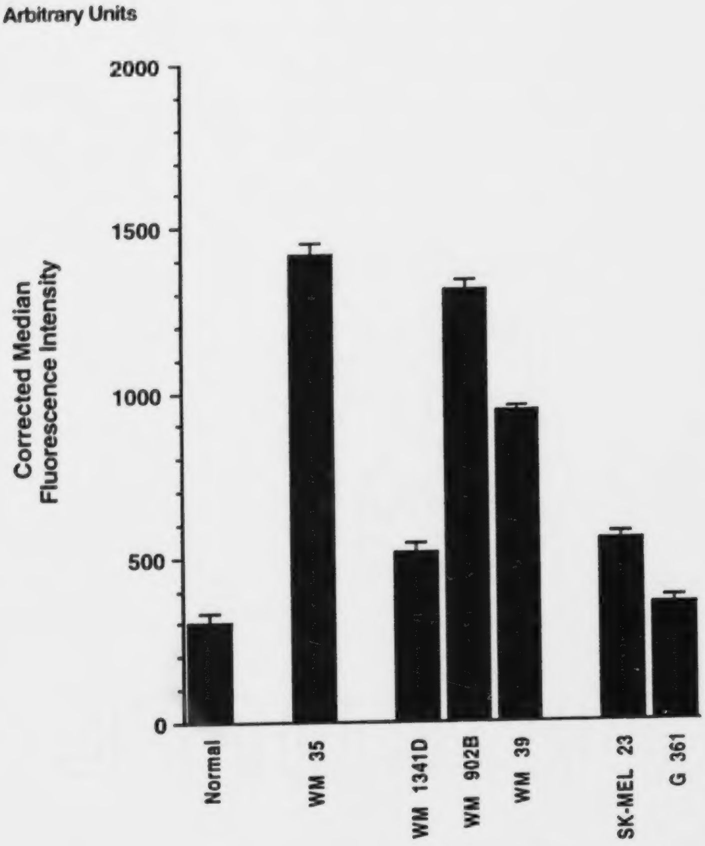


FIGURE IV-1: Corrected Median Fluorescence Intensity measuring ß1-Integrin Expression on Normal and Malignant Melanocytes

The median fluorescence intensity was significantly greater than negative control cells for all cell lines. The median fluorescence intensity is greatest for the RGP cell line, WM 35, and becomes progressively less with each advancing phase of tumour progression. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.

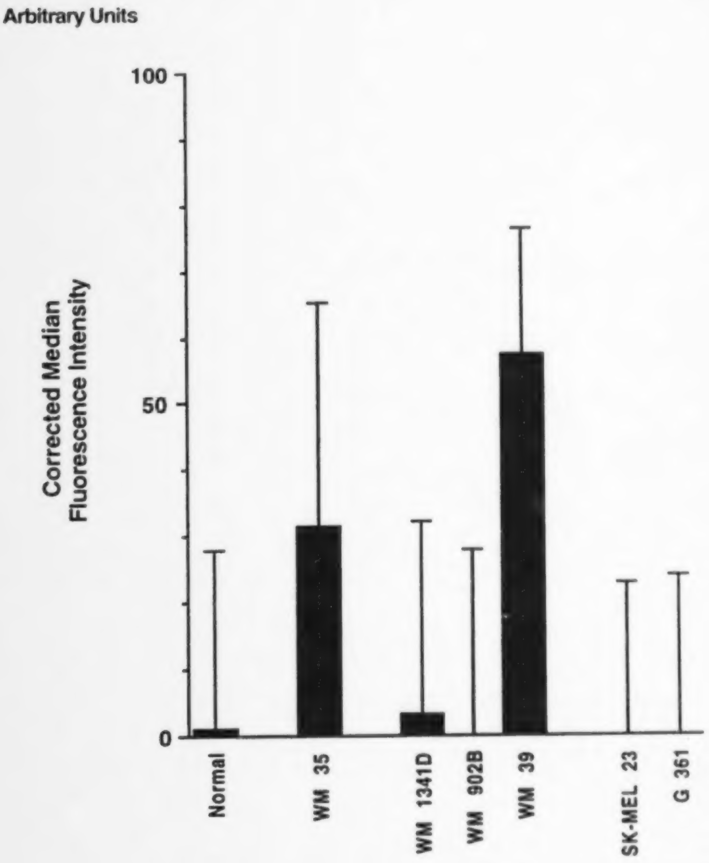


FIGURE IV-2: Corrected Median Fluorescence Intensity measuring ß3-Integrin Expression on Normal and Malignant Melanocytes

Only the VGP cell line, WM 39, has a median fluorescence intensity significantly greater than control cells. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.

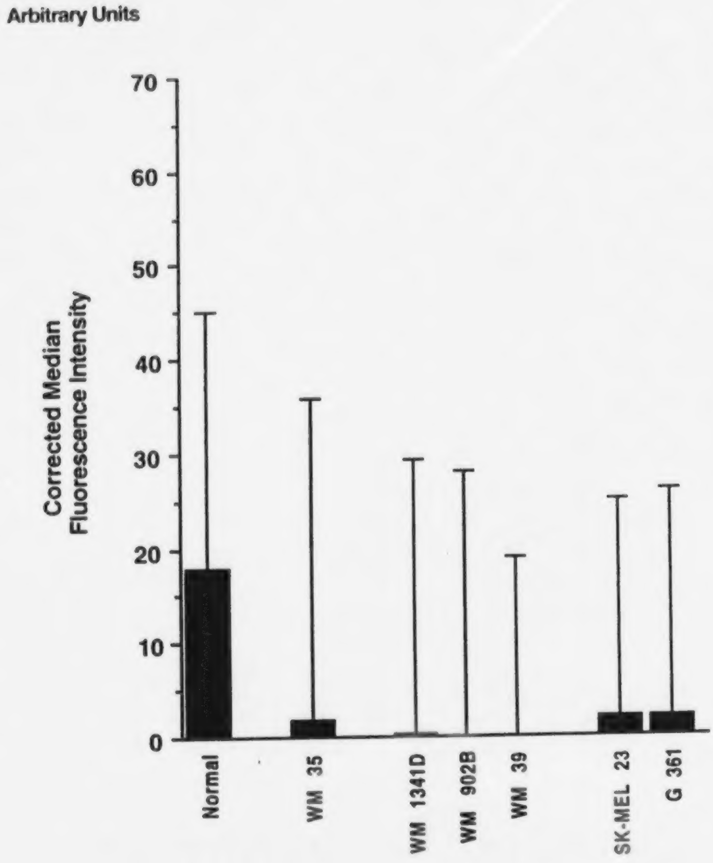


FIGURE IV-3: Corrected Median Fluorescence Intensity measuring ß4-Integrin Expression on Normal and Malignant Melanocytes

Only normal melanocytes had a median fluorescence intensity approaching detectable levels. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.

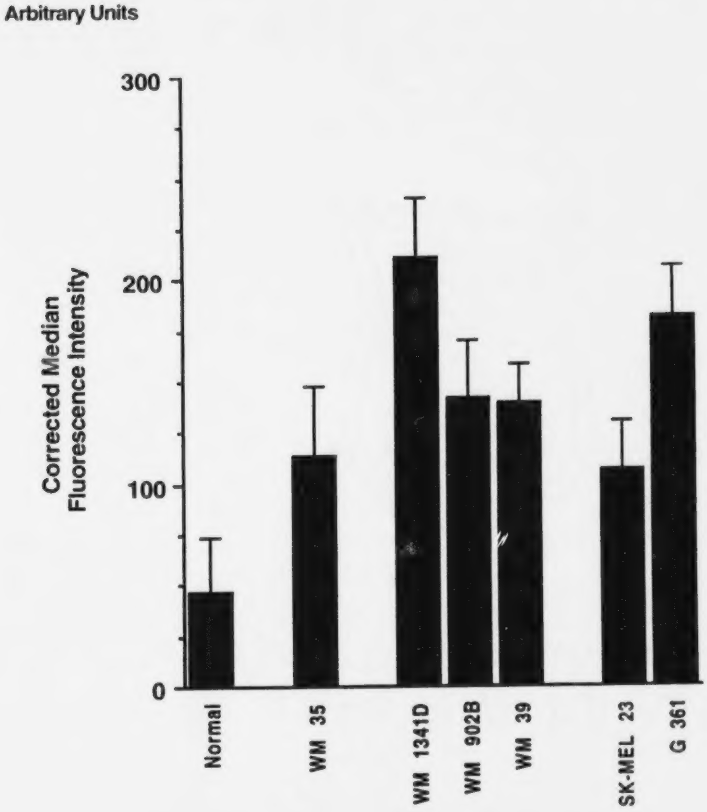


FIGURE IV-4: Corrected Median Fluorescence Intensity measuring α2-Integrin Expression on Normal and Malignant Melanocytes

The median fluorescence intensity values was significantly greater than control cells. Median fluorescence intensity increases from normal melanocytes to a maximum with VGP, and is maintained through the MGP cell lines. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.

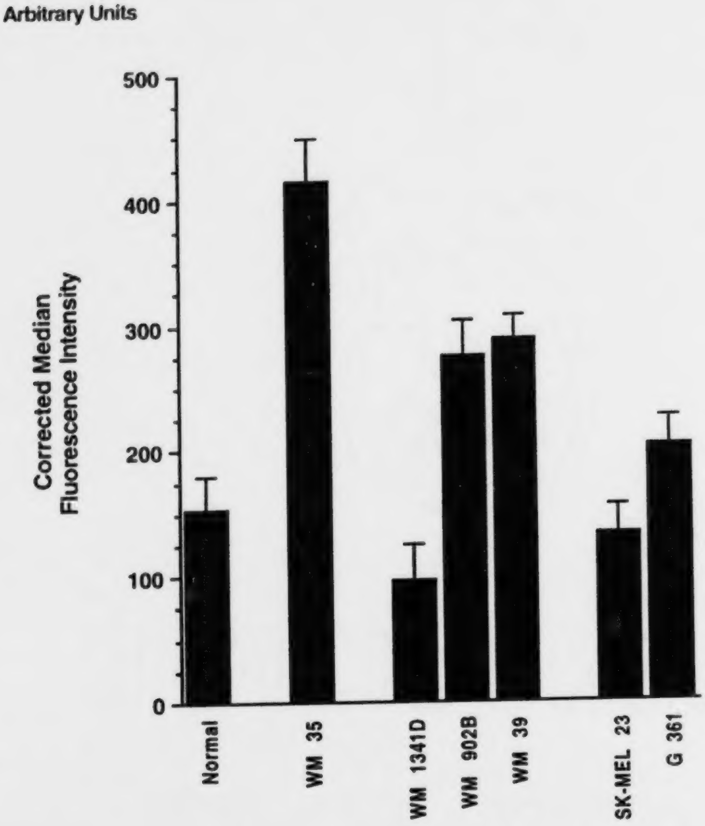


FIGURE IV-5: Corrected Median Fluorescence Intensity measuring α3-Integrin Expression on Normal and Malignant Melanocytes

The median fluorescence intensity values was significantly greater than control cells. Median fluorescence intensity peaks with the RGP cell line, then decreases with progressive phases of tumour progression. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.

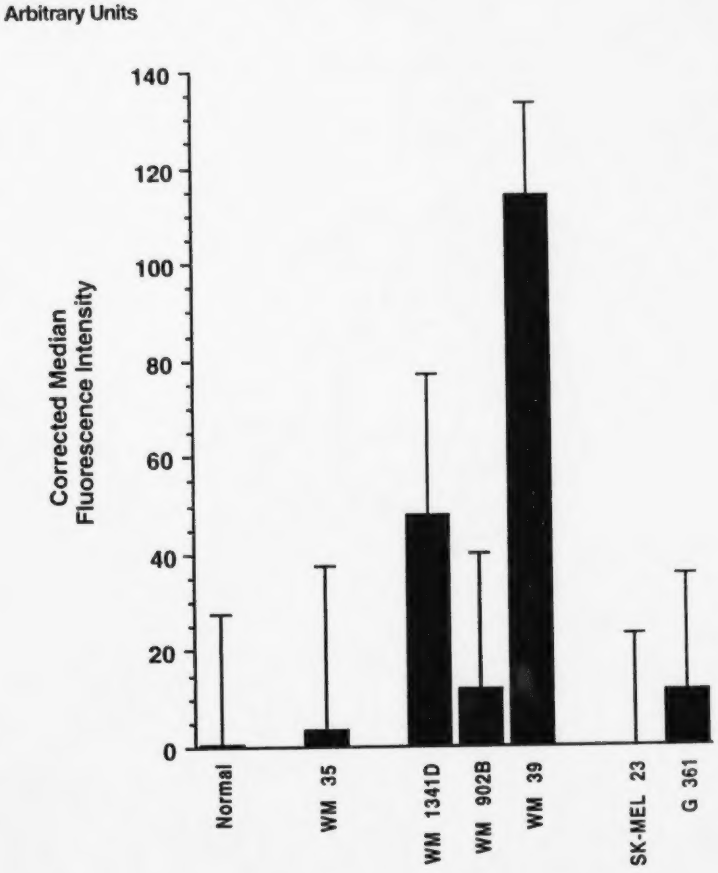


FIGURE IV-6: Corrected Median Fluorescence Intensity measuring α4-Integrin Expression on Normal and Malignant Melanocytes

Only the VGP cell lines expressed median fluorescence intensities at a significant level. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.

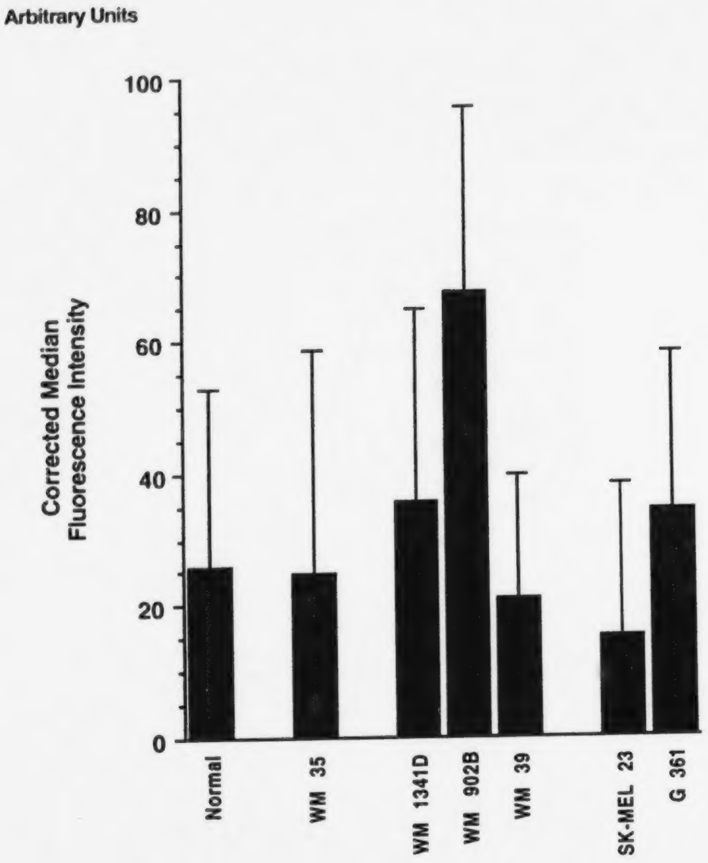


FIGURE IV-7: Corrected Median Fluorescence Intensity measuring α5-Integrin Expression on Normal and Malignant Melanocytes

Median fluorescence intensities remain stable through all phases of tumour progression. Only WM 902B cells had a median fluorescence intensity that approached significant levels greater than control cells. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.

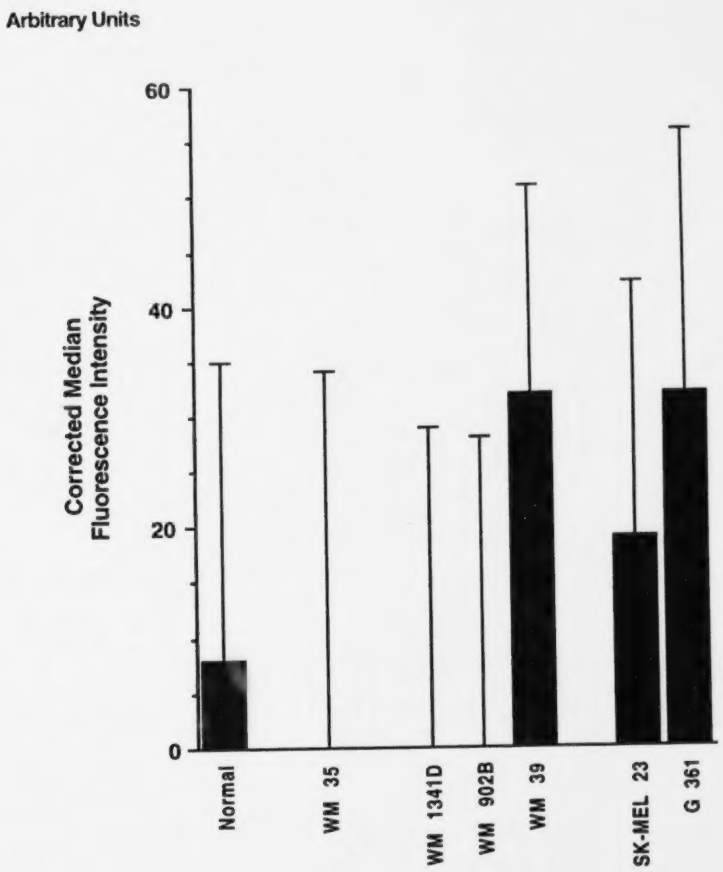
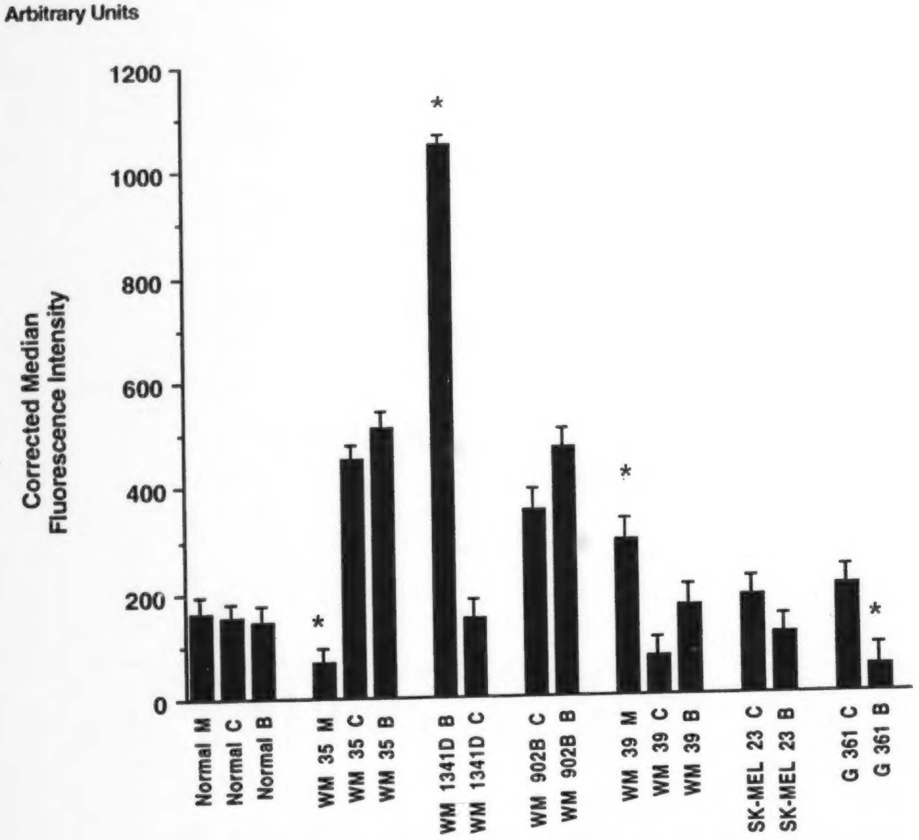


FIGURE IV-8: Corrected Median Fluorescence Intensity measuring αv-Integrin Expression on Normal and Malignant Melanocytes

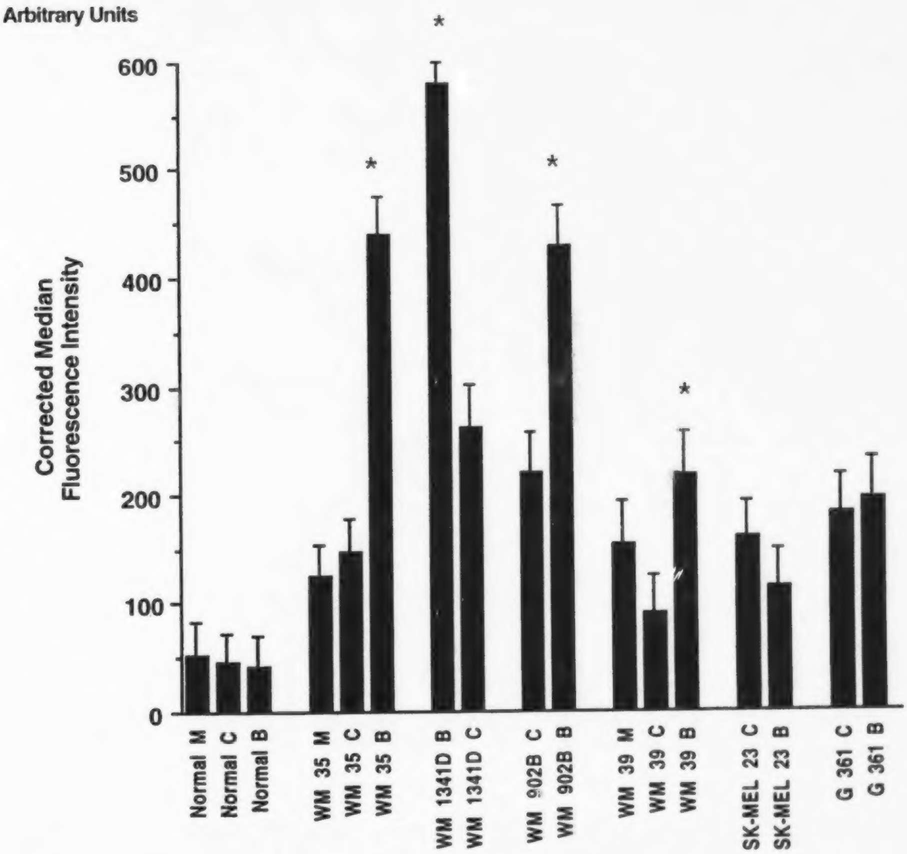
Only the VGP cell line WM 39, and the MGP cell lines had median fluorescence intensities approaching significant levels. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.



$p < 0.05$

FIGURE IV-9: Effect of BRDU and α-MSH on Median Fluorescence Intensity measuring α3-integrin expression on Normal and Malignant Melanocytes

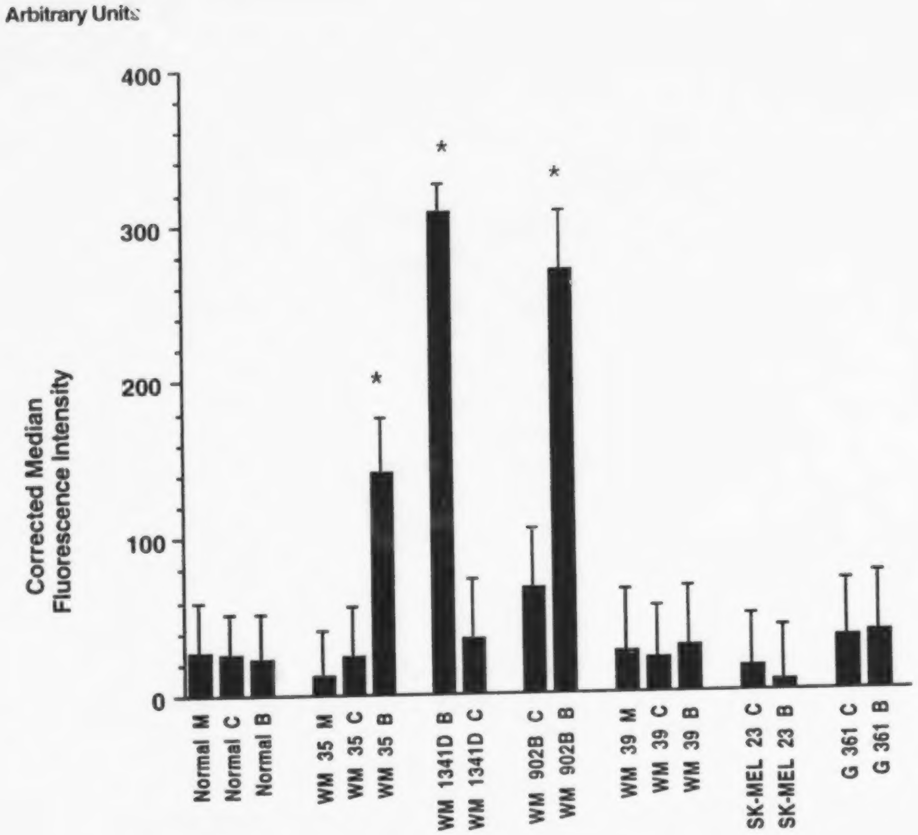
Cells exposed to either BRDU (B) or α-MSH (M) are positioned relative to the unexposed control (C) cells according to their effect on cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the median fluorescence intensities of exposed cells are more similar to the intensities of unexposed cells in the adjacent tumour phase. Also, the degree of change in intensity is greatest for the RGP cell line, WM 35, and the early VGP cell line, WM 1341D, and decreases with advancing phases of tumour progression. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.



$p < 0.05$

FIGURE IV-10: Effect of BRDU and α-MSH on Median Fluorescence Intensity measuring $\alpha 2$-integrin expression on Normal and Malignant Melanocytes.

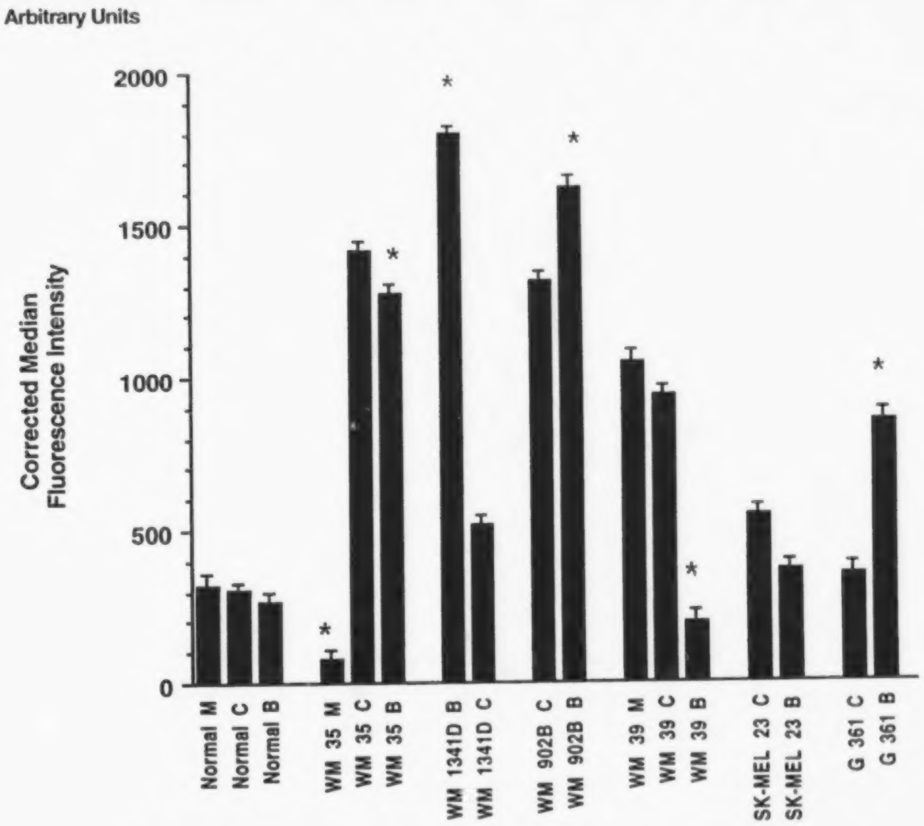
Cells exposed to either BRDU (B) or α-MSH (M) are positioned relative to the unexposed control (C) cells according to their effect on cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the median fluorescence intensities of exposed cells are more similar to the intensities of unexposed cells in the adjacent tumour phase. Also, the degree of change in intensity is greatest for the RGP cell line, WM 35, and the early VGP cell line, WM 1341D, and decreases with advancing phases of tumour progression. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.



$p < 0.05$

FIGURE IV-11: Effect of BRDU and α-MSH on Median Fluorescence Intensity measuring α5-integrin expression on Normal and Malignant Melanocytes

Cells exposed to either BRDU (B) or α-MSH (M) are positioned relative to the unexposed control (C) cells according to their effect on cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the median fluorescence intensities of exposed cells are more similar to the intensities of unexposed cells in the adjacent tumour phase. Also, the degree of change in intensity is greatest for the RGP cell line, WM 35, and the early VGP cell line, WM 1341D, and decreases with advancing phases of tumour progression. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.



$p < 0.05$

FIGURE IV-12: Effect of BRDU and α-MSH on Median Fluorescence Intensity measuring ß1-integrin expression on Normal and Malignant Melanocytes

Cells exposed to either BRDU (B) or α-MSH (M) are positioned relative to the unexposed control (C) cells according to their effect on cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the median fluorescence intensities of exposed cells are more similar to the intensities of unexposed cells in the adjacent tumour phase. Also, the degree of change in intensity is greatest for the RGP cell line, WM 35, and the early VGP cell line, WM 1341D, and decreases with advancing phases of tumour progression. Corrected median fluorescence intensity is the difference between the experimental fluorescence intensity and the fluorescence intensity of cells labelled with the PE-conjugated secondary antibody only.

REFERENCES

Albelda SM. Role of integrins and other cell adhesion molecules in tumor progression and metastasis. Lab. Invest. 68:4-17, 1993.

Albelda SM, SA Mette, DE Elder, R Stewart, L Damjanovich, M Herlyn, C Buck. Integrin distribution in malignant melanoma: association of the ß3 subunit with tumor progression. Cancer Res. 50:6757-64, 1990.

Albino AP. The role of oncogenes and growth factors in progressive melanoma-genesis. Pigment Cell Res. Suppl 2:199-218, 1992.

Anichini A, M Mortarini, E Berti, et al. Multiple VLA antigens on a subset of melanoma clones. Human Immunol. 28:119-122, 1990.

Eisinger M, Marko O. Selective proliferation of normal human epidermal melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc.Natl.Acad.Sci.(USA). 79:2018-2022, 1982.

Gailit JO, RAF Clark. Integrins. Adv.Dermatol. 8:129-153, 1993.

Hara H MH Lee, H Chen, D Luo, K Jimbow. Role of gene expression and protein synthesis of tyrosinase, TRP-1, Lamp-1, and CD63 in UVB-induced melanogenesis in human melanomas. J. Invest. Dermatol. 102:495-500, 1994.

Halaban R, FD Alfano. Selective elimination of fibroblasts from cultures of normal human melanocytes. In Vitro 20:447-450, 1984.

Houghton AN, FX Real, LJ Davis, C Cordon-Cardo, LJ Old. Phenotypic heterogeneity of melanoma: relation to the differentiation program of melanoma cells. J. Exp. Med. 164:812-829, 1987.

Humphries MJ, AP Mould, DS Tuckwell. Dynamic aspects of adhesion receptor function - integrins both twist and shout. BioEssays 15:391-397, 1993.

Hynes RO. Integrins: versatility, modulation, and signalling in cell adhesion. Cell 69:11-25, 1992.

Kaur P, WG Carter. Integrin expression and differentiation in transformed human epidermal cells is regulated by fibroblasts. J.Cell Sci. 103:755-763, 1992.

Kramer RH, M Vu, Y-F Cheng, DM Ramos. Integrin expression in malignant melanoma. Cancer and Metastasis Rev. 10:49-59, 1991.

Lynch SA, BN Bouchard, S Vijayasanadhi, H Yuasa, AN Houghton. Antigens of melanocytes and melanoma. Cancer Metastasis Rev. 10:141-50, 1991.

Mortarini R, A Anichini. Form adhesion to signalling: roles of integrins in the biology of human melanoma. Melanoma Res. 3:87-97, 1993.

Ruoslahti E, FG Giancotti. Integrins and tumor cell dissemination. Cancer Cells 1:119-126, 1989.

Williams MJ, PE Hughes, TE O'Toole, MH Ginsberg. The inner world of cell adhesion: integrin cytoplasmic domains. Trends Cell Biol. 4:109-112, 1994.

Zambruno G, PC Marchisio, A Melchiori, S Bondanza, R Cancedda, M DeLuca. Expression of integrin receptors and their role in adhesion, spreading and migration of normal human melanocytes. J. Cell Sci. 105:179-190, 1993.

V. ADHESION BY NORMAL AND MALIGNANT MELANOCYTES DERIVED FROM DIFFERENT STAGES OF TUMOUR PROGRESSION TO EXTRACELLULAR MATRIX COMPONENTS: EFFECTS OF DIFFERENTIATION-INDUCING AGENTS 5-BROMO-DEOXYURIDINE AND α-MELANOCYTE STIMULATING HORMONE ON CELL-SUBSTRATE ADHESION

INTRODUCTION

Integrins regulate cell motility, and the control of growth and differentiation of multiple cell types in the epidermis [McCarthy et al, 1988; Ruoslahti and Pierschbacher, 1987]. Normal melanocytes and malignant melanoma cells may display different adhesion abilities to extracellular matrix proteins. This study is an attempt to describe the adhesion strengths between normal and malignant melanocytes under a variety of conditions.

In order to determine whether normal melanocytes and malignant melanocytes derived from different phases of tumour progression differed in their adhesion to extracellular matrices, we used a semi-quantitative adhesion assay to measure the adhesion strength against a uniform detachment force. A quantitative method measuring the functional adhesion strength of a molecule will help to clarify how a particular cell adhesion molecule controls these functions. A method that is reproducible and sensitive enough to measure small adhesion differences is desirable. Lotz and colleagues [1991] described a centrifugation method applying a reproducible vertical detachment force against cells plated onto a substrate. We have previously reported the development of a modified method for a quantitative attachment assay for adherent cells [J.Cell.Biochem. 16F:152, 1992 (abstr)]. Our method uses a nitrocellulose strip to collect the nonadherent cells, and a colorimetric assay based on the cell's ability to reduce a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide (MTT), and form a blue formazan product by the mitochondrial enzyme, succinate dehydrogenase [McClay et al, 1981]. The method represents a simple, rapid, and sensitive way to determine the quantity of live cells attached to a substratum in microwell plates for multiple cell attachment assays.

This study demonstrates that the degree of cell-substrate adhesion to four common extracellular matrix substrates is dependent upon the growth phase of tumour progression. Furthermore, cell-substrate adhesion is lowest for the radial growth phase melanoma, WM 35, and greatest for the vertical growth phase melanomas. However, cell-substrate adhesion can be regained in the radial growth phase melanoma, WM 35, following exposure to BRDU. In general, the degree of adhesion strength is decreased during tumour progression. Taken together with the results previously discussed (see above), these data would suggest that the radial growth phase melanoma, WM 35, expresses integrins in an inactivated form, and that the integrins may be reactivated following exposure to BRDU.

MATERIALS AND METHODS

Materials

MTT, fibronectin, laminin, collagen type IV, ethylenediamine tetraacetic acid (EDTA), and trypsin/EDTA were obtained from Sigma (St. Louis, MO); Nunc Polysorp F-16 wells, Dulbecco's Modified Essential Medium, and other cell culture reagents from Gibco (Montreal, PQ); nitrocellulose, and the silicone gasket and pressure plate which was fashioned from a large gel electrophoresis apparatus from Bio-Rad (Mississauga, ON); MCDB 153 media from Clonetics (San Diego, CA); DMSO (spectrophotometric grade) from Aldrich (Milwaukee, WI); the nonfat skim milk powder from Carnation (Lemington, ON); the 0.22 µm syringe filter was obtained from Millipore (Mississauge, ON); and the ELISA microwell plate reader came from SLT industries (Salzberg, Austria).

Cell Lines and Culture Conditions:

The cell lines and culture conditions were as described in Chapter III (p. 39).

Preparation of Extracellular Matrix Components

The microwells in 96-well plates were coated with one of the following substrates dissolved in 0.1 M phosphate buffer (pH 7.4): fibronectin (10 µg/ml); laminin (10 µg/ml); collagen Type I (10 µg/ml); and collagen Type IV (10 µg/ml). The wells were loaded with 50 µl of each solution and incubated at 37°C. for 60 min. After rinsing twice with 10 mM EDTA in PBS, all nonspecific sites were blocked with 10% nonfat milk powder incubated for 60 min at 37°C. Milk was selected as the blocking agent of choice after testing several other agents (see Appendix V). The wells were rinsed twice with 10 mM EDTA in PBS, and a final rinse with PBS before the addition of the pigment cells.

MTT-Microculture Tetrazolium Assay

An MTT stock solution was prepared at a concentration of 5 mg/ml in 0.1 M phosphate buffer and stored at 4°C. The working concentration was 0.5 mg/ml, diluted with RPMI 1640 media. The stock solution was filtered through a 0.22 μm filter to remove any formazan crystals.

The microwells containing the cells were gently aspirated using a 30-gauge needle and 100 μl of MTT working solution was added to the wells containing cells. After incubation for 3 hr at 37°C., the MTT solution was aspirated with a 30-gauge needle and 100 μl DMSO was added. The plates were placed on a rotary shaker at 150 rpm for 45 min. Absorbance at 540 nm was read,on a microplate reader, and the values in the test wells were corrected for the values in the blank wells containing DMSO alone. Results were expressed as the mean and standard deviation of six-duplicate determinations.

Centrifugation Cell Detachment Assay

The method was a modification of the method reported by Lotz et al [1991]. The pigment cells were harvested with 10mM EDTA in PBS. The cells were pelleted by centrifugation at 200xg at room temperature in a Beckman TJ-6 centrifuge, and resuspended in serum-free media. The cells were plated on precoated microwells at $5x10^4$/100 μl. The cells were then briefly centrifuged (200xg) at room temperature for 2 min in the TJ-6 centrifuge with a swinging bucket rotor in order to bring cells into contact with the plate surface. The cells were then incubated for the indicated time periods in an incubator (37°C, 5% CO_2).

The wells were gently filled to the top with fresh media prewarmed to 37°C, and a nitrocellulose membrane strip premoistened with media was placed over the wells, taking care to exclude all air bubbles. The silicone rubber gasket and pressure plate were placed over the nitrocellulose, and clamped using a bulldog stationery clamp. The gasket was fashioned from the silicone rubber strip that sealed the bottom of the gel plates in the casting stand. The pressure plate was made from a single thick gel plate spacer used to make large format gels. Both the gasket and the spacer were cut into two lengths of 10 cm each. The apparatus was then placed in the TJ-6 centrifuge in an inverted position with the nitrocellulose on the bottom of the bucket, and centrifuged for 8 min at three different speeds; 500 rpm (54xg), 1000 rpm (212xg), or 2000 rpm (854xg). The apparatus was disassembled and the strip carefully peeled from the wells, placed over a new 96-well plate and the impressions caused by the wells of the original plate containing the detached cells were aligned with the microwells and punched into the wells using a flame-burnished glass rod with an outside diameter equal to the inside diameter of the microwell. The MTT microculture tetrazolium assay was performed on the cells contained in the two microwell plates (see above).

Statistical Analysis

Experiments were performed in duplicate, and the means and their standard deviations were calculated from multiple replicates performed with each experiment. Statistically significant differences between the mean values were determined using the Student's t-distribution. Values for $p \leq 0.05$ were considered statistically significant.

RESULTS

Characterization of the Centrifugation Cell Detachment Assay

Effect of Incubation Time

The time course for formazan production by the attached melanocytes was examined. Dye formation was very rapid, with optimal formation at 3 hr (Figure V-1). All later experiments used an incubation period of 3 hr.

Effect of Cell Density on Dye Formation

The effect of cell number on dye formation was assessed to determine whether the absorbance of the formazan product was directly proportional to the number of attached pigment cells. The absorbance was linearly correlated to the number of cells within the range of $5x10^3$ to $1x10^5$ cells per well (Figure V-2). This is in agreement with the findings of Slater et al [1963], who found that the formazan absorbance was linear to cell number within this range.

Effect of Nitrocellulose Membrane on Assay

Since detached cells were collected onto a nitrocellulose membrane strip, it was important to determine whether the nitrocellulose membrane would affect either the cell's ability to form a formazan product, or the absorbance value following solution of the nitrocellulose membrane by DMSO. Cells collected on the nitrocellulose membrane were able to form the dye product, and the sum of the two compartments closely approximated the total number of cells (Figure V-3).

The DMSO-soluble nitrocellulose membrane did not alter the optimal absorbance wavelength of 540 nm for detecting the formazan product (Figure V-4) and therefore, no correction is required for nitrocellulose membrane.

Effect of Blocking Agents

Several protein solutions were tested to determine the optimal agent to block nonspecific binding sites. These included bovine serum albumin (BSA) [1-10% (w/v)], ovalbumin (OVA) [1-10% (w/v)], nonfat skim milk powder [1-10% (w/v)], and lambda carageenan [0.2-2% (w/v)] dissolved in 0.1 M phosphate buffer (pH 7.4). The percent adhesion of total cell number was assessed after 1 hr incubation at 37°C on blocked plastic. The optimal blocking agents were 5% BSA, and 10% nonfat milk powder (Figure V-5). Remaining experiments were done using milk powder.

Cell-Substrate Adhesion of Normal and Malignant Melanocytes

Normal and malignant melanocytes demonstrated a wide range of adhesion strength to all substrates tested (Tables V-6 to V-9). In general, all cells tested, except the metastatic cell lines SK-MEL 23 and G 361, had low adhesion strength to blocked plastic substrates. After correcting for baseline adhesion to blocked plastic, normal melanocytes, in general, showed a greater adhesion strength to all substrates than any of the malignant cell lines tested (Figures V-6 to V-9). Normal melanocytes adhered best to fibronectin, followed by laminin, collagen type IV, and type I, in order of decreasing adhesion. The radial growth phase melanoma cell line, WM 35, had a low adhesion strength to all substrates tested, as did the melanotic, metastatic growth phase melanoma cell line, SK-MEL 23. The vertical growth phase melanoma cell lines, WM 39, WM 902B showed an adhesion strength intermediate between the normal melanocytes and the low adherent WM 35 and SK-MEL 23. Fibronectin was the best substrate, followed by the collagens, with laminin being the substrate with the lowest adhesion strength. The amelanotic metastatic growth phase melanoma cell line, G 361 showed an adhesion strength between the low adhesion WM 35 and SK-MEL 23, and the vertical growth phase melanomas. The collagens were the best substrate for these cells, followed by laminin, with fibronectin being the substrate with the lowest adhesion strength. Thus, the pattern of cell-substrate adhesion may be dependent upon the growth phase of the tumour progression, although the wide heterogeneity in total adhesion for the same detachment force makes the trend difficult to detect.

Close examination of cell-substrate adhesion for different cell lines show that the changes in adhesion strength are substrate-specific. For example, adhesion to fibronectin showed that RGP cells decreased adhesion strength to a level about one-fifth of normal melanocytes, whereas the VGP cell lines increased adhesion strength more than two-fold above normal melanocytes. Cells in the metastatic growth phase had very low adhesion strength compared to normal melanocytes. A similar pattern of adhesion strength was also observed using Type I and Type IV Collagen as a substrate. When laminin was used as substrate, reduced adhesion strength was observed with the RGP cell line. However, while adhesion strength did increase with the VGP cell lines, it was still lower than that observed with normal melanocytes. The metastatic cell lines again demonstrated a low adhesion strength.

The reduced adhesion strength of the radial growth phase WM 35 to all substrates would imply that these cells would not bind well to any basement membrane component, usually laminin and collagen type IV. This may explain why radial growth phase melanoma cells are commonly observed within the epidermis, and not attached to the basement membrane. Once the cells invade the underlying dermis, the cells now must be able to adhere and migrate through an extracellular matrix rich in collagen type I and fibronectin. This increased adhesion strength by the VGP cell lines would support this finding (Table V-1). Finally, metastatic cells may not require significant adhesion strength once in a secondary tumour. Also, a low adhesion state would permit cells to easily detach from the primary tumour and embolize. The reduced adhesion strength observed with the metastatic cell lines tested supported this possibility.

Despite the general trends, there was substantial heterogeneity between cell lines of the same growth phase. For example, all VGP cell lines had high adhesion abilities to fibronectin, and reduced adhesion abilities to laminin. Yet, WM 39 and WM 902B had high adhesion strengths to the collagens, whereas WM 1341D demonstrated a reduced adhesion strength.

Cell-Substrate Adhesion of Malignant Melanocytes following Exposure to 5-Bromodeoxyuridine

All cell lines tested, except WM 39, demonstrated changes in cell-substrate adhesion following exposure to BRDU for 7 days (Figures V-10 to V-13). Previous experiments (see Chapter III) had demonstrated that BRDU transformed the cellular differentiation phenotype of all cells, except WM 1341D, into a less differentiated phenotype. As seen in Figures V-10 to V-13, the changes in cell-substrate adhesion for each particular cell type reflected the changing trends seen between tumour progression phases. For example, the degree of cell-substrate adhesion for each extracellular matrix tested had increased when RGP and VGP melanomas are compared. Similarly, as the RGP cell line WM 35 became less differentiated following BRDU exposure, the degree of cell-substrate adhesion also increased for all substrates tested. As another example, the VGP cell line WM 902B became less differentiated following BRDU exposure. Yet, the WM 902B cell line had a decreased adhesion ability to fibronectin, and the collagens, which reflected the change in cell-substrate adhesion observed when the VGP and MGP melanomas were compared. Thus, the observed changes in cell-substrate adhesion between phases of tumour progression may be attributed to changes which occurred during the loss of differentiation by the melanocytes. However, the exact mechanisms by which these changes occur are not known.

Cell-Substrate Adhesion of Malignant Melanocytes following Exposure to α-Melanocyte Stimulating Hormone

The radial growth phase melanoma cell line, WM 35, and the vertical growth phase melanoma cell line, WM 39 were examined for differences in cell-substrate adhesion following exposure to α-melanocyte stimulating hormone. As discussed previously (Chapter III), both WM 35 and WM 39 acquire a cellular phenotype consistent with a more differentiated phenotype. Similar to the effect observed with BRDU, the degree of change in cell-substrate adhesion strength following exposure to α-melanocyte stimulating hormone reflected the changes seen between tumour phases (Figures V-14 to V-17). For example, the RGP WM 35 cell line had a greater adhesion strength to fibronectin, laminin, and Collagen type I, while the adhesion strength to Collagen type IV decreased. This increase in the degree of cell-substrate adhesion is comparable to the increase in the degree of cell-substrate adhesion observed between normal melanocytes and the radial growth phase melanoma. Additionally, the WM 39 cell line showed a decrease in adhesion strength to all substrates, which was the trend observed in cell-substrate adhesion when moving from the VGP to the RGP tumour phase. Thus, the degree of cell-substrate adhesion appeared to be dependent upon the phase of tumour progression, and the state of differentiation.

DISCUSSION

This study showed that the cell-substrate adhesion could be reproducibly measured by a centrifugation assay. Cell adhesion is functionally defined as the ability of cells to bind to each other, or to a substrate. Until recently, this capacity has been qualitatively defined by applying a fluid shear force to cells during washing [McClay et al, 1981]. This method lacks the sensitivity to detect minor alterations in adhesion, because cell adhesion is a dynamic process. In this study, we have developed a rapid, simple test for detecting small changes in adhesion strength. We have shown that this assay can detect minute differences in adhesion between normal and malignant melanocytes under a variety of media conditions, and multiple samples can be run simultaneously.

The present results should be compared cautiously with those of previous investigators [Kueng et al, 1989; Margis et al, 1989]. Biophysically, the fluid shear forces produced by the cell washing method are of a different direction and duration than a prolonged (8 min) physical traction in a direction perpendicular to the plane of adhesion during centrifugation. The cell may be able to better withstand this method of detachment shear, because of the cytoskeletal organization. Perhaps, a more accurate term for this assay would be a vertical detachment assay, rather than an adhesion assay, to account for these differences.

This assay offers several advantages over previous methods [Kueng et al, 1989; Margis et al, 1989]. The assay is simple and uses common laboratory equipment, obviating the need for radionuclide facilities and monitoring equipment. The tetrazolium salt can only be converted to formazan by intact mitochondria, so that only viable cells are measured. Other cell staining methods, such as Coomassie brilliant blue R-250 [Margis et al, 1989], detect both living and dead cells, which may affect percent cell adhesion determinations. Furthermore, cell counting methods are either labour-intensive (manual counting) or require specialized equipment (automated counting). The MTT method has been compared to the [^{51}Cr] release assay, and also shows a linear relation between signal and cell number [Heo et al, 1990]. However, unlike the [^{51}Cr] release assay, the level of sensitivity is much higher and can accurately detect as few as 200 cells, allowing the detection of as few as several hundred cells with minimal background.

Integrins are pluripotent in their ability to bind to several extracellular matrix components. This multiple functionality provides the cell with a wide repertoire of potential ligands for binding with the extracellular matrix. However, evidence has shown that integrin-ligand specificity is cell-specific, and somewhat restrictive [Mortarini et al, 1991]. In other words, a particular cell may use a potentially multifunctional integrin to bind to only one extracellular matrix component, such as only fibronectin, or laminin. Putative ligands for the integrins are as follows: $\alpha 2\beta 1$, collagen; $\alpha 3\beta 1$, laminin, and possibly fibronectin; $\alpha 4\beta 1$, alternative forms of fibronectin; $\alpha 5\beta 1$, fibronectin; $\alpha v\beta 3$, fibronectin, laminin; and $\alpha 6\beta 4$, laminin.[Zambruno et al, 1993].

However, very few instances could be found where changes in integrin expression could predict changes in cell-substrate adhesion to the substrate ligand. For example, α2β1 is thought to be the major collagen receptor, yet in the SK-MEL 23 cell line, a 58% increase in α2β1 integrin expression by non-exposed SK-MEL 23 cells over BRDU-exposed SK-MEL 23 cells, could not account for the 60 - 88% decrease in adhesion ability by these cells to collagen. Several possible explanations include the presence of an undetermined integrin receptor being responsible for SK-MEL 23 adhesion to collagen, and the α2β1 integrin is inactivated; alternatively, the α2β1 integrin is activated, but the function is prevented by a counter-regulatory signal from another signal transduction pathway. Finally, the α2β1 receptor is active and bound to the collagen, but the intracellular cytoskeletal organization is disrupted, resulting in a dysfunctional adhesion complex. It is not known whether these, or any other, explanation is responsible for the observed incongruity.

The degree of cell-substrate adhesion as assessed in the assay shows that this adhesion is sensitive to the state of cellular differentiation. Several cell lines from different tumour progression phases had comparable levels of integrin expression, yet widely variable degrees of substrate adhesion. Cell adhesion may require more than the presence of integrin subunits on the cell surface. One possible explanation for these differences could be that the integrins may be in a different activation state, which would either increase or decrease the degree of adhesion. Studies of integrin activation on lymphocytes and platelets have shown that integrin subunits can exist in an inactivated form, with poor binding affinity for its ligand in a resting state [Ginsberg et al, 1992; Juliano, 1987; McEver, 1992; Quaranta et al, 1991; Schweighoffer et al, 1992]. Stimulation of lymphocytes by growth factors, or platelet degranulation can cause a temporary activation of integrin avidity for its ligand, greatly increasing adhesive strength [Schweighoffer et al, 1992]. It is conceivable that malignant transformation may alter the activation state of one or more integrin subunits [Juliano, 1987], which is translated into observed changes in adhesion. Studies using monoclonal antibodies which recognize integrin subunits in various states of activation may be useful in addressing this issue [Stupack et al, 1992].

An alternative explanation for the differences in adhesion between normal melanocytes, G361 and SK-MEL 23 involves the alterations in the cytoskeletal-integrin-substrate adhesion complex. Since integrins are responsible for integrating the extracellular matrix with the cytoskeleton, any alterations in the cohesive structure linking the cytoplasmic tail of the integrin subunit with the cytoskeleton will affect the tensile strength of the bond. The composition of the integrin-cytoskeletal complex is not completely defined yet, but it is arguable that mutations in any one of these molecules during malignant transformation will alter the ability of the complex to withstand any tensile load. The composition of the cytoskeletal-integrin complex in the early differentiated G361 clone may be better adapted to withstand this load. The cytoskeletal-integrin complex of late differentiated SK-MEL 23 clone may not be as well organized as G361 or normal melanocytes, leading to the decreased adhesion to all substrates tested. Co-localization studies using double immunofluorescence, and biochemical isolation of each component protein may characterize the components of this adhesion complex.

A third explanation which may include the above concepts would be an altered signal transduction pathway present in the malignant cells, which would either inactivate the integrin's binding ability, or interfere with the formation of the integrin-cytoskeleton adhesion complex. Since the process of adhesion is apparently very complex and dynamic, and further studies examining this area will require extremely well defined substrates and cell systems, likely involving transfected adhesion components which have been engineered using site-directed mutagenesis. While some of these studies are just now being reported, the evidence is far too preliminary to speculate. However, the varying degree of cell-substrate adhesion exhibited by the radial growth phase melanoma, WM 35, makes this cell line a potentially useful model with which to study cellular control mechanisms involved with adhesion complex formation can be studied.

Table V-1: **Changes in Cell-Substrate Adhesion during Transitions between Phases of Tumour Progression**

	Epidermis	Dermis	Distant Organs
	CIV, LN, Epiligrin	CI, FN	FN, LN
Substrate	N→RGP	RGP→VGP	VGP→MGP
FN	↓↓	↑↑	↓
LN	↓↓	↑	↓
COLL I	↓↓	↑	↓
COLL IV	↓↓	↑	↓

N = Normal
RGP = Radial growth phase
VGP = Vertical growth phase
MGP = Metastatic growth phase
FN = Fibronectin
LN = Laminin
COLL I, CI = Collagen type I
COLL IV, CIV = Collagen type IV
↑ = increased adhesion strength
↓ = decreased adhesion strength

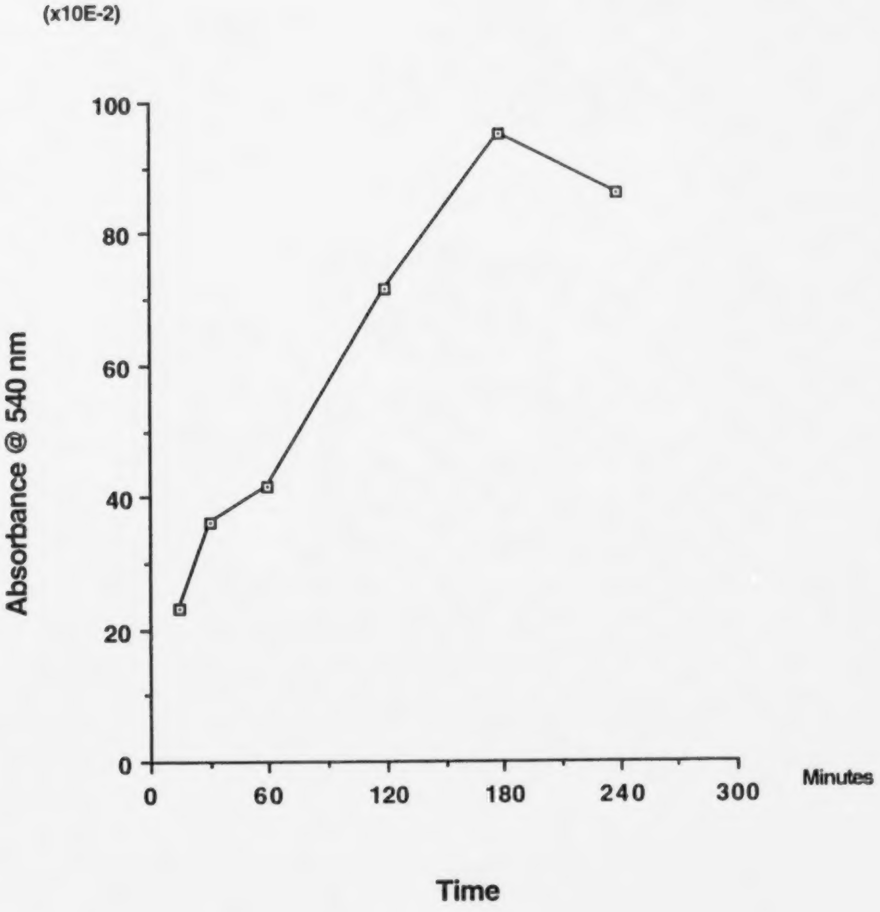


FIGURE V-1: Effect of Incubation Time on Formazan Dye Formation by Melanocytes

Optical absorbance at 540 nm of solubilized formazan produced by 10^5 normal melanocytes using DMSO as the solvent. Three hour incubations were used for all subsequent experiments.

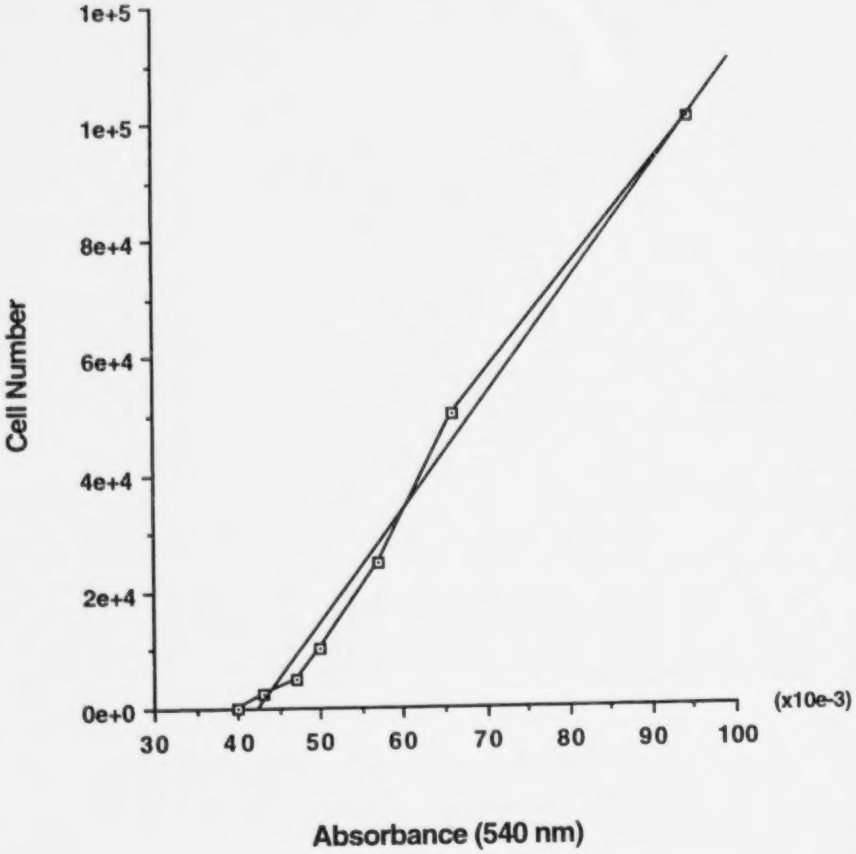


FIGURE V-2: Effect of Cell Density on Formazan Dye Formation by Melanocytes

Optical absorbance at 540 nm of solubilized formazan produced by various numbers of normal melanocytes using DMSO as the solvent. The absorbance was linear within the range of 5000 to 100,000 cells.

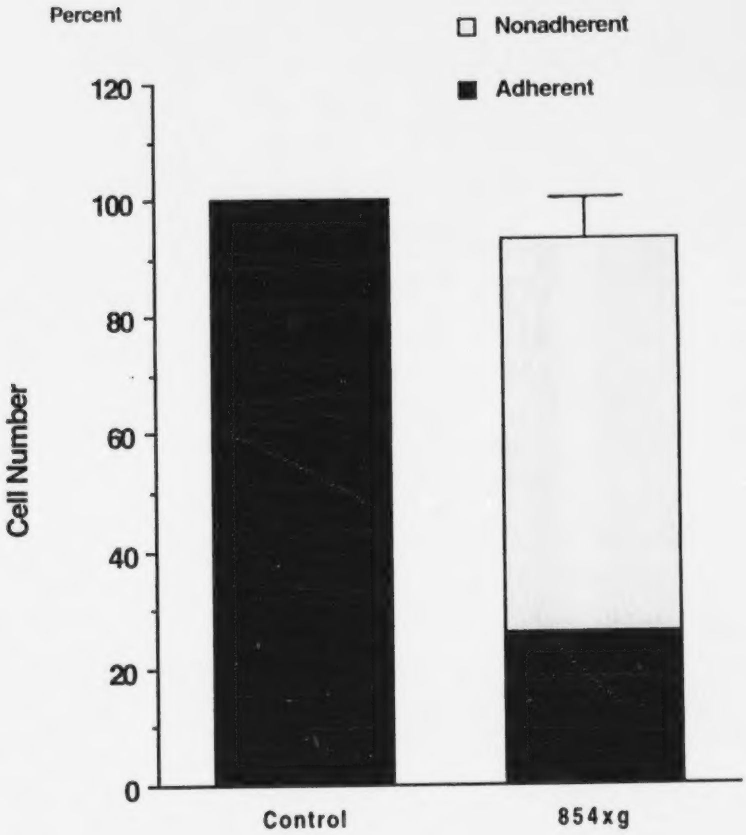


FIGURE V-3: Effect of Nitrocellulose Membrane on Cell Viability of Melanocytes during Centrifugation

Almost all the normal melanocytes recovered from the centrifugation assay following maximal centrifugation (854xg) were detected by the MTT colourimetric assay, indicating that the cells collected on the nitrocellulose membrane were viable during the assay period. The control cells were not subjected to any centrifugation prior to the MTT assay.

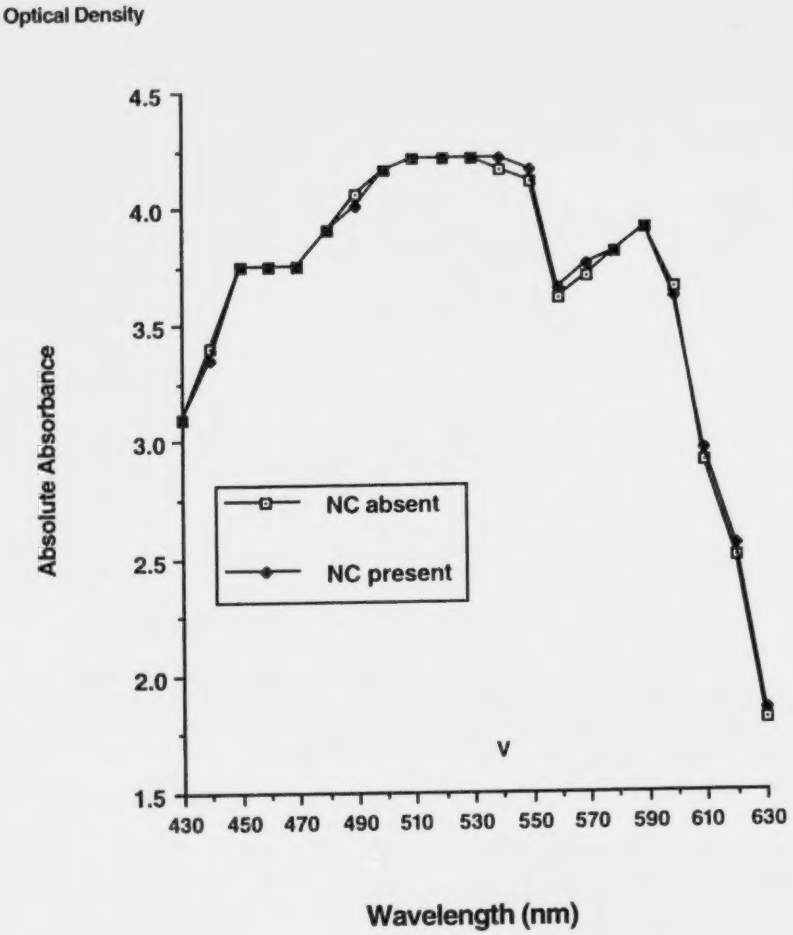


FIGURE V-4: Effect of Nitrocellulose Membrane on Formazan Dye Detection using Spectrophotometric Analysis

No significant shift in optical absorbance was noted by the presence of nitrocellulose membrane in solution with DMSO. Arrow represents the optical absorbance used for measurement assay.

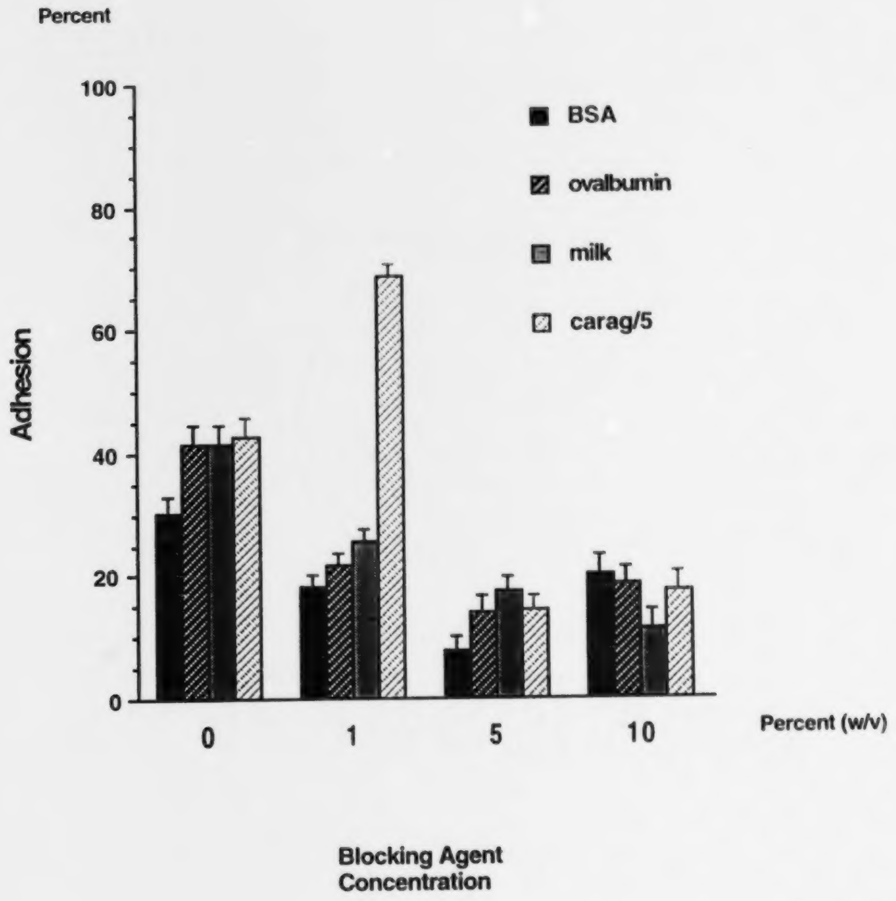


FIGURE V-5: Effect of Blocking Agents on Cell Adhesion Assay

Plastic wells were coated for 1 hour at 37°C. with various concentrations of bovine serum albumin (BSA), ovalbumin, nonfat milk, or lambda carageenan. Percent adhesion of normal melanocytes following 1 hour incubation at 37°C was determined using the centrifugation assay at 212xg. 5% BSA and 10% nonfat milk were the best blocking agents.

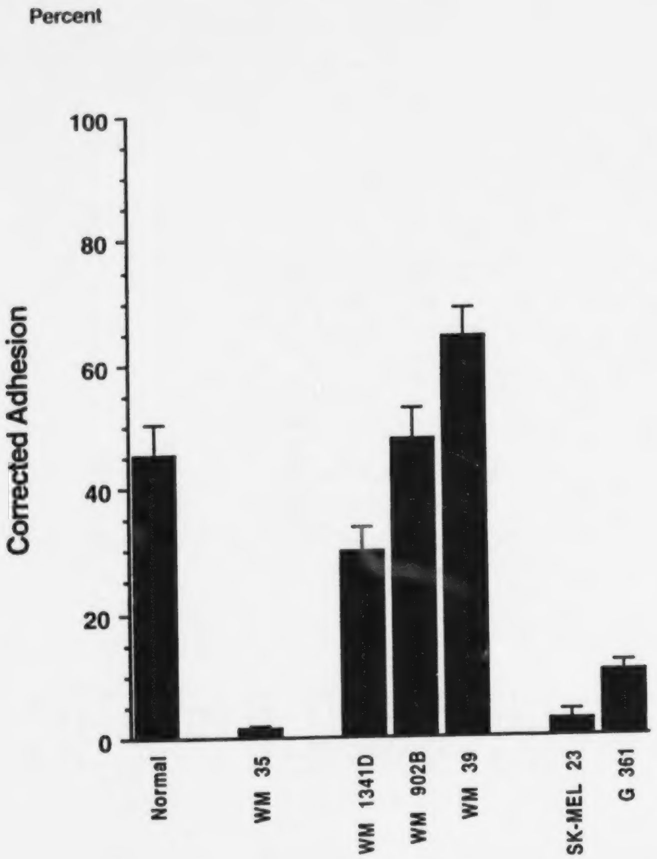


FIGURE V-6: Adhesion to Fibronectin by Normal and Malignant Melanocytes

WM 35 adhered poorly to this substrate, as did the MGP cell lines, whereas the VGP cell lines had significant adhesion strength. Centrifugation force applied: 212xg. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.

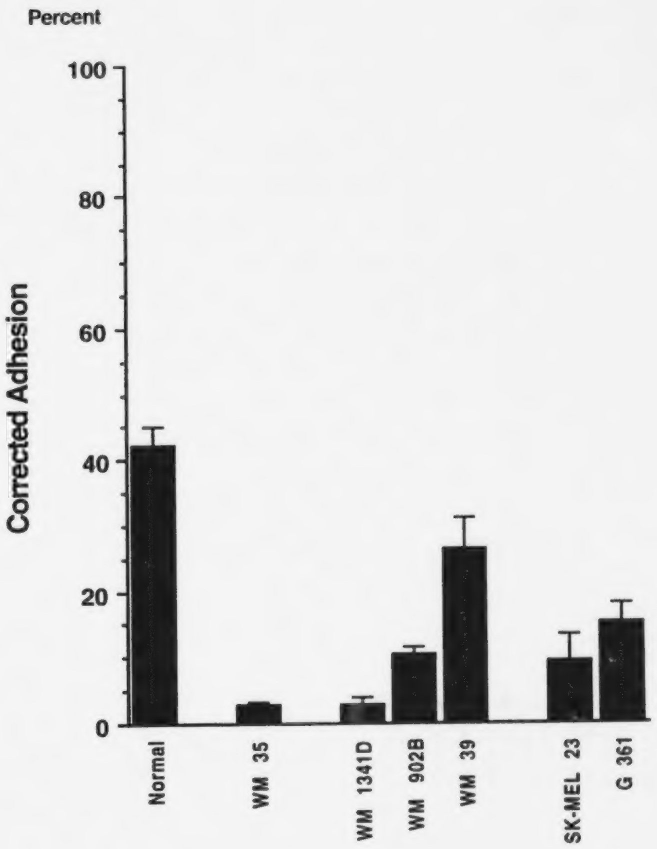


FIGURE V-7: Adhesion to Laminin by Normal and Malignant Melanocytes

Normal melanocytes adhered well to this substrate, whereas WM 35 had very low adhesion strength. Both the VGP and MGP cell lines had a minimal adhesion strength to this substrate. Centrifugation force applied: 212xg. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.

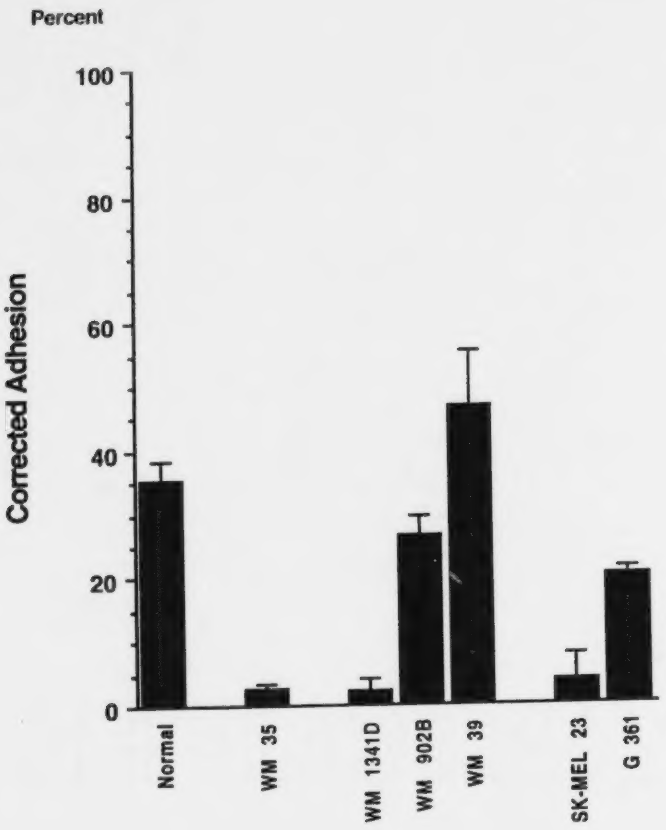


FIGURE V-8: Adhesion to Collagen Type I by Normal and Malignant Melanocytes

WM 35 adhered poorly to this substrate, as did the MGP cell lines, whereas the VGP cell lines had significant adhesion strength. Centrifugation force applied: 212xg. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.

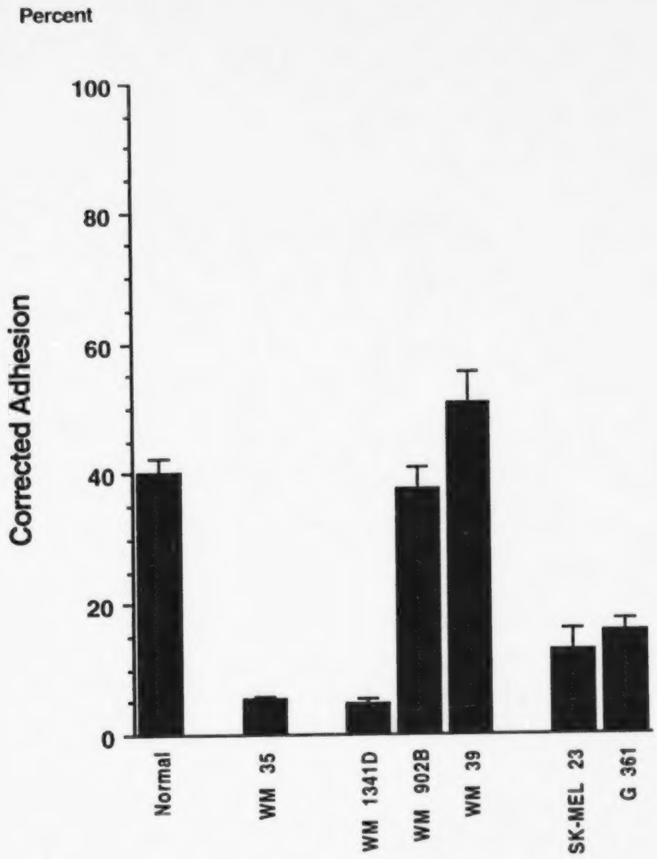
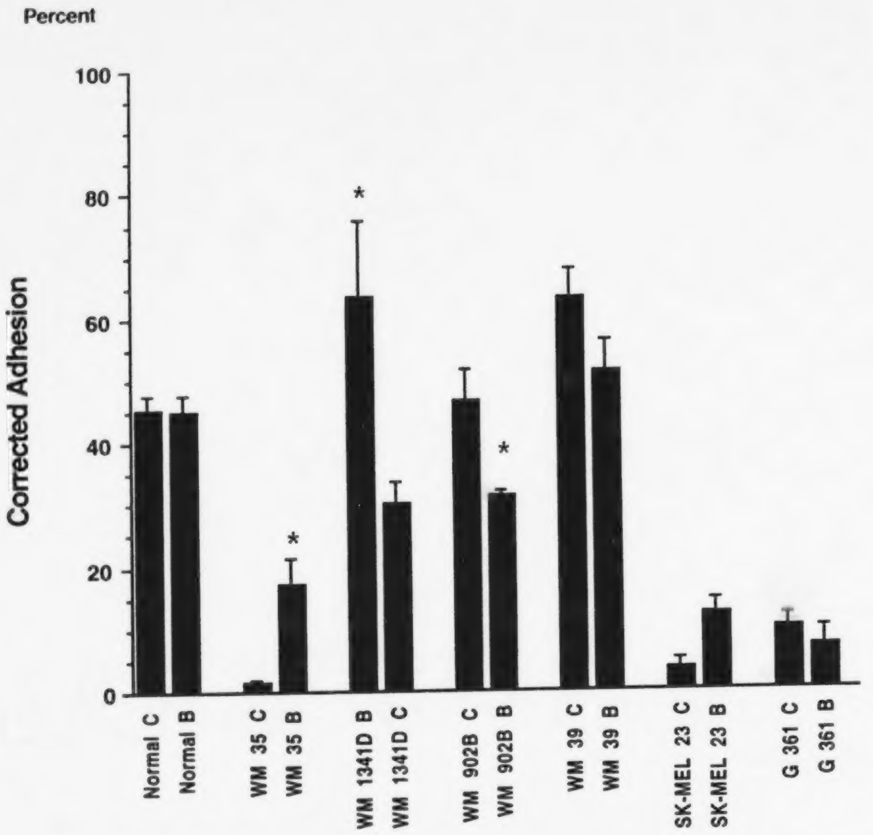


FIGURE V-9: Adhesion to Collagen Type IV by Normal and Malignant Melanocytes

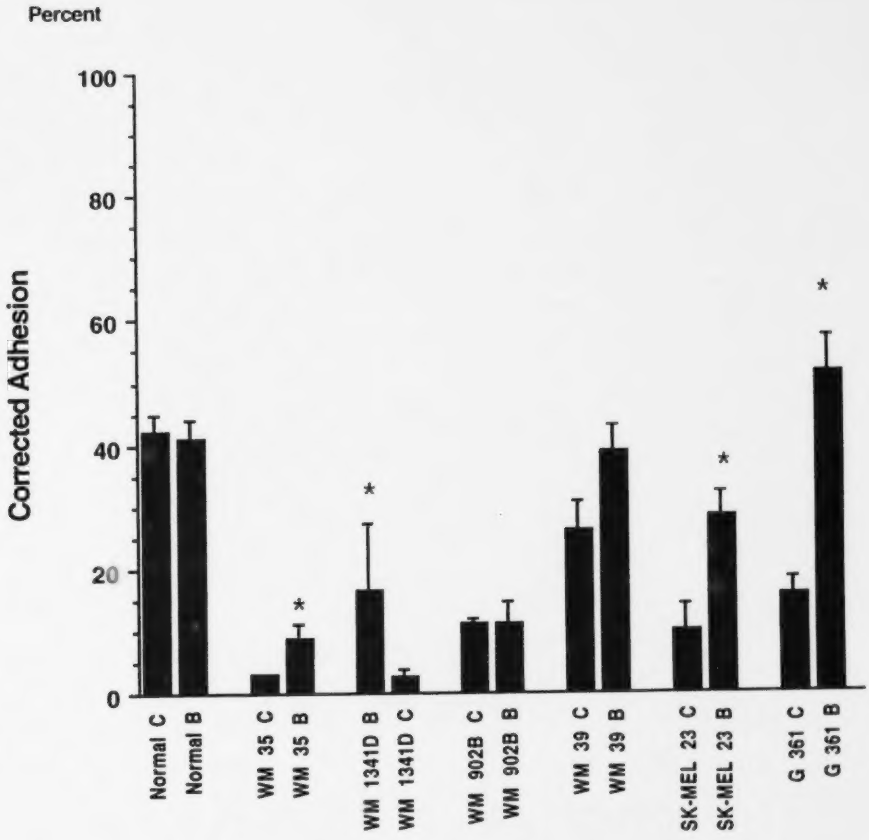
WM 35 adhered poorly to this substrate, as did the MGP cell lines, whereas the VGP cell lines had significant adhesion strength. Centrifugation force applied: 212xg. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE V-10: Effect of BRDU on Adhesion to Fibronectin by Normal and Malignant Melanocytes

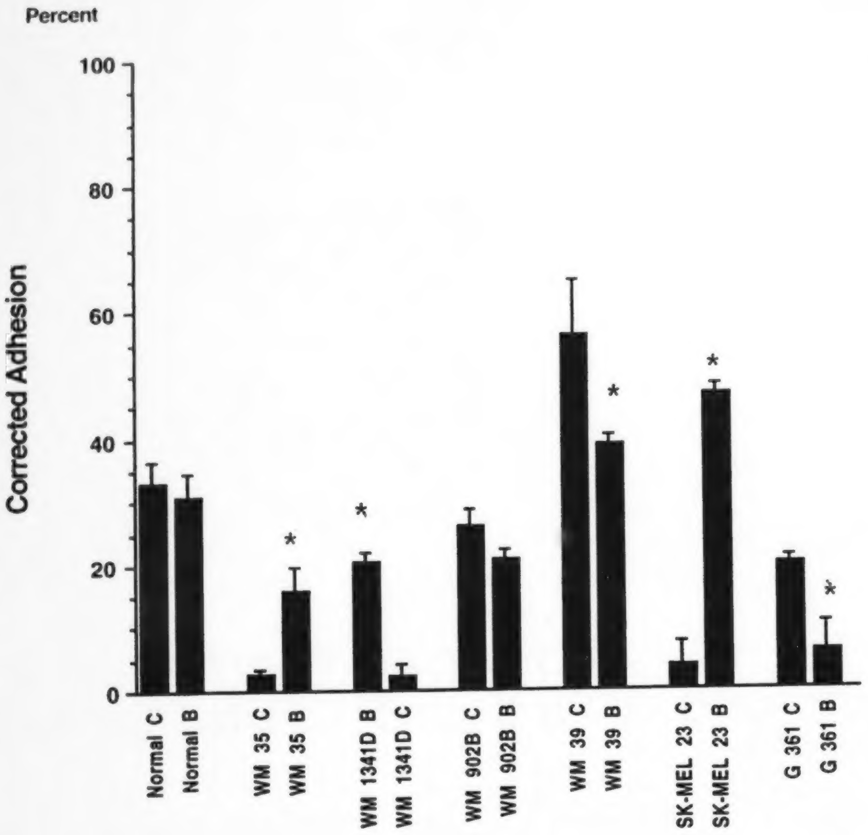
BRDU-exposed cells (B) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the adhesion strength of BRDU-exposed cells changes to a level similar to that of the control cells in the adjacent phase of tumour progression. Also, the degree of change in cell adhesion strength is greatest in the RGP and early VGP cells, and diminishes with the advancing phases of tumour progression. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE V-11: Effect of BRDU on Adhesion to Laminin by Normal and Malignant Melanocytes

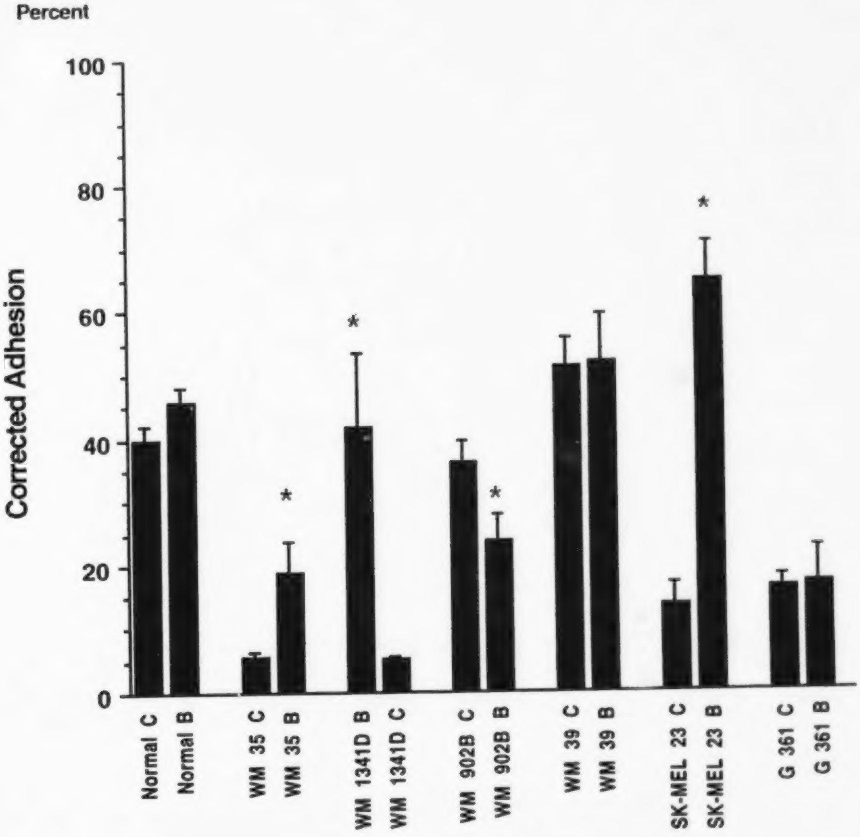
BRDU-exposed cells (B) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the adhesion strength of BRDU-exposed cells changes to a level similar to that of the control cells in the adjacent phase of tumour progression. Also, the degree of change in cell adhesion strength is greatest in the RGP and early VGP cells, and diminishes with the advancing phases of tumour progression. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE V-12: Effect of BRDU on Adhesion to Collagen Type I by Normal and Malignant Melanocytes

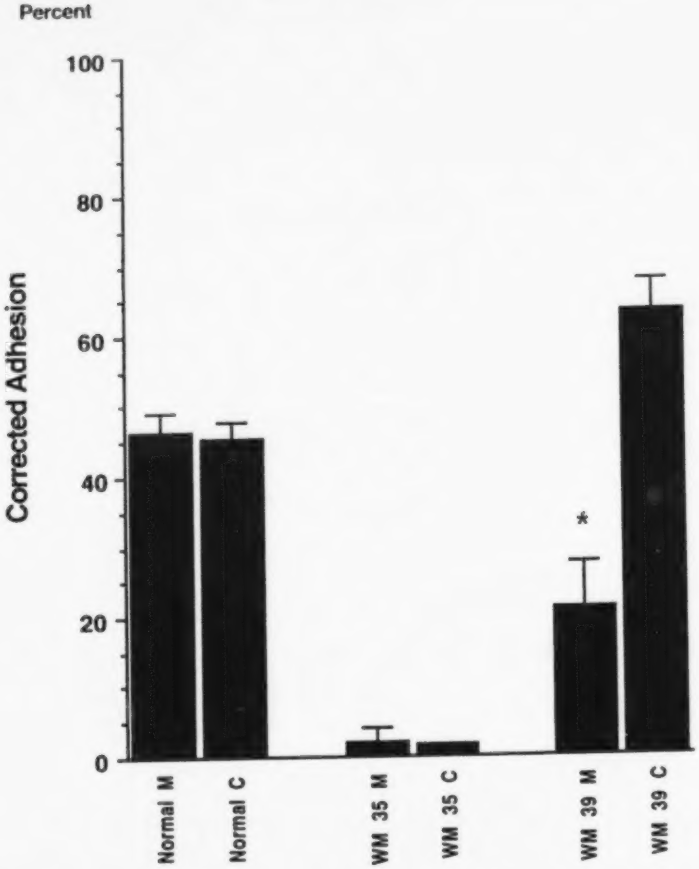
BRDU-exposed cells (B) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the adhesion strength of BRDU-exposed cells changes to a level similar to that of the control cells in the adjacent phase of tumour progression. Also, the degree of change in cell adhesion strength is greatest in the RGP and early VGP cells, and diminishes with the advancing phases of tumour progression. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE V-13: Effect of BRDU on Adhesion to Collagen Type IV by Normal and Malignant Melanocytes

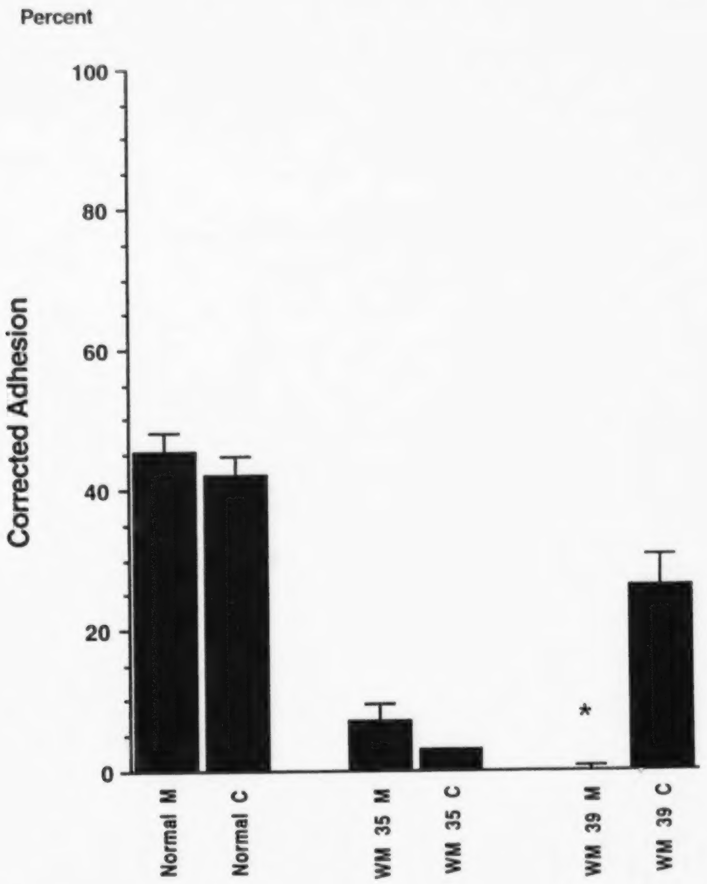
BRDU-exposed cells (B) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the adhesion strength of BRDU-exposed cells changes to a level similar to that of the control cells in the adjacent phase of tumour progression. Also, the degree of change in cell adhesion strength is greatest in the RGP and early VGP cells, and diminishes with the advancing phases of tumour progression. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE V-14: Effect of α-MSH on Adhesion to Fibronectin by Normal and Malignant Melanocytes

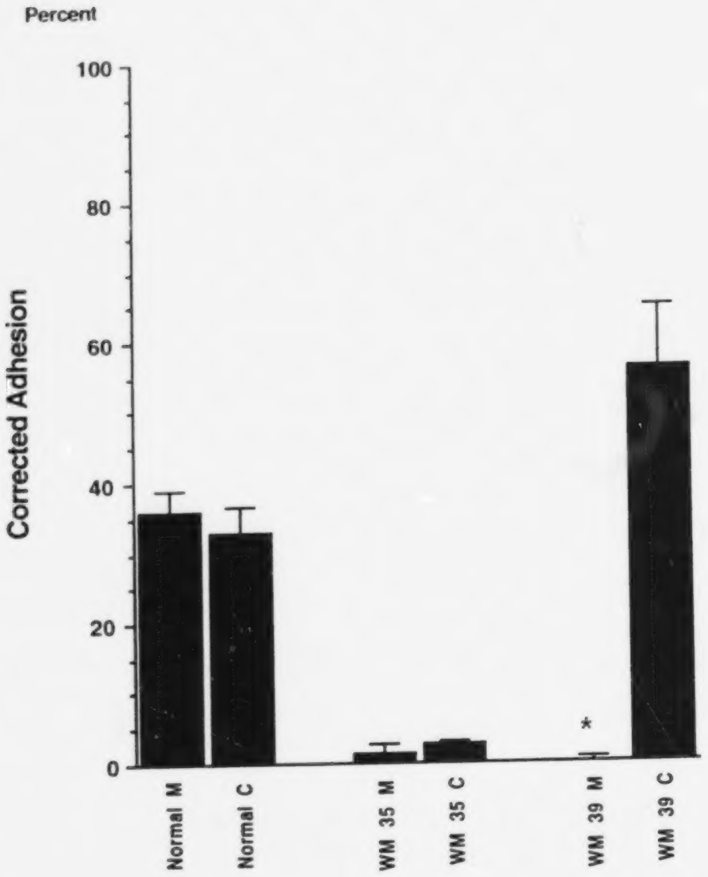
α-MSH-exposed cells (M) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the adhesion strength of α-MSH-exposed cells changes to a level similar to that of the control cells in the adjacent phase of tumour progression. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE V-15: Effect of α-MSH on Adhesion to Laminin by Normal and Malignant Melanocytes

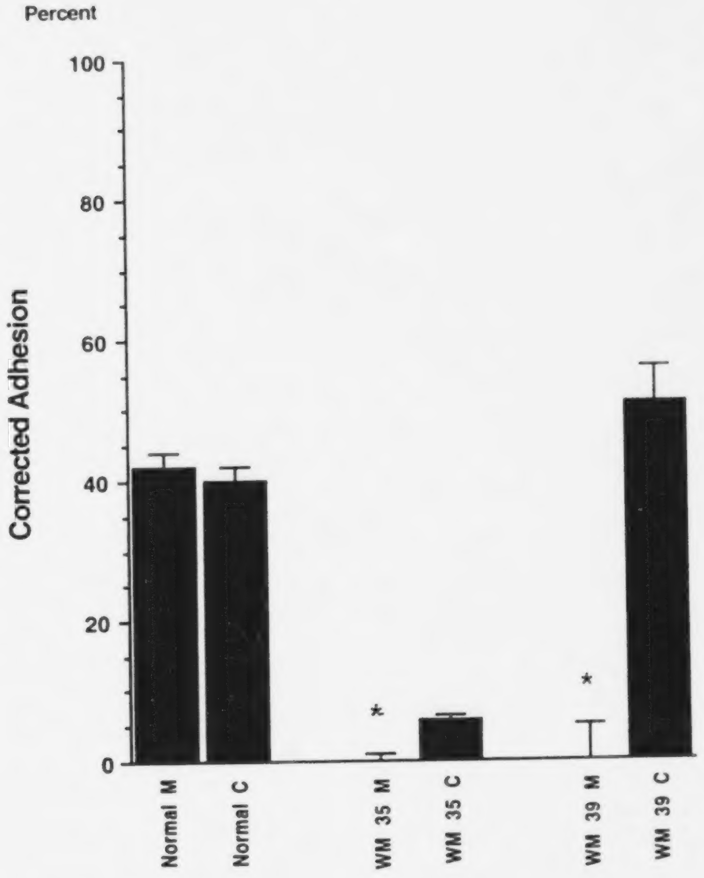
α-MSH-exposed cells (M) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the adhesion strength of α-MSH-exposed cells changes to a level similar to that of the control cells in the adjacent phase of tumour progression. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE V-16: Effect of α-MSH on Adhesion to Collagen Type I by Normal and Malignant Melanocytes

α-MSH-exposed cells (M) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the adhesion strength of α-MSH-exposed cells changes to a level similar to that of the control cells in the adjacent phase of tumour progression. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE V-17: Effect of α-MSH on Adhesion to Collagen Type IV by Normal and Malignant Melanocytes

α-MSH-exposed cells (M) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. In general, the adhesion strength of α-MSH-exposed cells changes to a level similar to that of the control cells in the adjacent phase of tumour progression. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.

BIBLIOGRAPHY

Albelda SM, SA Mette, DE Elder, R Stewart, L Damjanovich, M Herlyn, C Buck. Integrin distribution in malignant melanoma: association of the ß3 subunit with tumor progression. Cancer Res. 50:6757-64, 1990.

Albelda SM. Role of integrins and other cell adhesion molecules in tumor progression and metastasis. Lab. Invest. 68:4-17, 1993.

Bauer JS, CL Schreiner, FG Giancotti, E. Ruoslahti, RL Juliano. Motility of fibronectin receptor-deficient cells on fibronectin and vitronectin: collaborative interactions among integrins. J. Cell Biol. 116:477-487, 1992.

Brown PJ, RL Juliano. Selective inhibition of fibronectin-mediated cell adhesion by monoclonal antibodies to a cell-surface glycoprotein. Science 228:144-145, 1985.

Eisinger M, Marko O. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc.Natl.Acad.Sci.(USA) 79:2018-2022, 1982.

Gillies RG, N Diddier, M Denton. Determination of cell number in monolayer culutres Anal.Biochem. 159:109-113, 1986.

Ginsberg MH, X Du, EF Plow. Inside-out integrin signalling. Curr. Opin. Cell Biology 4:766-771, 1992.

Grzesiak JJ, GE Davis, D Kirchhofer, MD Pierschbacher. Regulation of α2ß1-mediated fibroblast migration on type I collagen by shifts in the concentrations of extracellular Mg^{+2} and Ca^{+2}. J.Cell Biol. 117:1109-1117, 1992.

Halaban R, FD Alfano. Selective elimination of fibroblasts from cultures of normal human melanocytes. In Vitro 20:447-450, 1984.

Halaban R. S Ghosh, P Duray, JM Kirkwood, AB Lerner. Human melanocytes cultured from nevi and melanomas. J. Invest. Dermatol. 87:95-101, 1986.

Henrotte JG. Genetic regulation of blood and tissue magnesium content in mammals. Magnesium 7:306-314. 1988.

Heo DS, J-G Park, K Hata, R Day, RB Herberman, TL Whiteside. Evaluation of tetrazolium-based semiautomatic colorimetric assay for measurement of human antitumor cytotoxicity. Cancer Research. 50:3681-3690, 1990.

Houghton AN, FX Real, LJ Davis, C Cordon-Cardo, LJ Old. Phenotypic heterogeneity of melanoma: relation to the differentiation program of melanoma cells. J. Exp. Med. 164:812-829, 1987.

Humphries MJ. The molecular basis and specificity of integrin-ligand interactions. J. Cell Sci. 97:585-592, 1990.

Juliano R. Membrane receptors for extracellular matrix macromolecules: relationship to cell adhesion and tumor metastasis. Biochim.Biophys.Acta. 977:261-278, 1987.

Kueng W, E Silber, U Eppenberger. Quantification of cells cultured on 96-well plates. Anal.Biochem. 182:16-19, 1989.

Lotz MM, CA Burdsal, HP Erickson, DR McClay. Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. J.Cell Biol, 109:1795-1805, 1989.

Mancianti ML, M Herlyn, D. Weil, J. Jambrosic U Rodick, D Becker, L Diamond, WH Clark, H Koprowski. Growth and phenotypic characteristics of human nevus cells in culture. J. Invest. Dermat. 90:134-141, 1988.

Margis R, R Borojevic. Quantification of attached cells in tissue culture plates and on microcarriers. Anal.Biochem. 181:209-211, 1989.

McCarthy JB, DF Sas, LT Furcht. (1988) In Clark RAF, PM Henson (eds.) Cell Biology of Wound Healing. Plenum, New York. pp.281-319.

McClay DR, GM Wessel, RB Marchase. Intercellular recognition: quantitation of initial binding events. Proc.Natl.Acad.Sci, (USA). 78:4975-4979, 1981.

McEver RP. Leucocyte-endothelial cell interactions. Curr. Opin. Cell Biology 4:840-849, 1992.

Miller RR, CA McDevitt. A quantitative microwell assay for chondrocyte cell adhesion Anal.Biochem. 192:380-383, 1991.

Mortarini R, A Anichini, G Parmiani. Heterogeneity for integrin expression and cytokine-mediated VLA modulcation can influence the adhesion of human melanoma cell to extracellular matrix proteins. Int.J.Cancer 47:551-559, 1991.

Quaranta V, JCR Jones. The internal affairs of an integrin. Trends Cell Biol. 1:2-4, 1991.

Ruoslahti E, MD Pierschbacher. New perspectives in cell adhesion: RGD and integrins. Science. 238:491-497, 1987.

Schweighoffer T, S. Shaw. Adhesion cascades: diversity through combinatorial strategies. Curr. Opin. Cell Biology. 4:824-829, 1992.

Slater TF, B Sawyer, U Strauli. Studies on succinate-tetrazolium reductase systems. III. Points of coupling of four different tetratzolium salts. Biochim.Biophys.Acta. 77:383-393, 1963.

Stupack DG, C Shen, JA Wilkins. Induction of $\alpha v\beta 3$ integrin-mediated attachment to extracellular matrix in $\beta 1$ integrin (CD29)-negative B cell lines. Exp.Cell Res. 203:443-448, 1992.

VI. EFFECT OF PERTURBATION OF INTEGRIN ACTIVITY ON CELL-SUBSTRATE ADHESION BY NORMAL AND MALIGNANT MELANOCYTES DERIVED FROM DIFFERENT STAGES OF TUMOUR PROGRESSION: ROLE OF FUNCTIONAL BLOCKING ANTI-INTEGRIN ANTIBODIES AND DIVALENT CATIONS.

INTRODUCTION

Biophysical evidence suggests that cell adhesion molecules can bind to their cognate receptor by bringing the two molecules close enough together to form a noncovalent chemical bond which is thermodynamically stable. In order for this event to occur, the binding site of the receptor must have the proper topography to mould with the "binding site" of the cognate receptor. It is assumed that cells which have the capacity to bind to a substrate must express sufficient numbers of receptors on the cell surface that are in the proper topographical conformation.

Conversely, if this close apposition between the receptor and its ligand is prevented, then significant binding cannot proceed. One method which could perturb cell adhesion would be to place a large molecule, such as an immunoglobulin molecule, at or near the binding site of the receptor. This interposition would block the binding site of the receptor though steric hinderance. Alternatively, if the receptor could be conformationally altered by changing the intramolecular stress so as to physically distort the binding site, the ability for close molecular apposition between the receptor and its ligand would be diminished, or lost altogether. The divalent metal cation binding site on integrins is thought to regulate the conformational shape of the integrin binding site [Gresiak et al, 1990]. Manganese is suggested by several investigators to provide the best conformational shape for integrin binding to Arginine-Glycine-Aspartate (RGD) depending epitopes [Grinnell et al, 1993]. Thus, several perturbation methods are available which may be able to modulate the binding effect attributed to integrins. Since changes in the extracellular environment, such as cell lysis, may produce different extracellular cation concentrations, this study is an attempt to investigate the effect of divalent cations on adhesion to substrates by normal and malignant melanocytes.

In order to determine whether specific integrins were responsible for cell-substrate adhesion by normal and malignant melanocytes derived from several phases of tumour progression, the degree of cell-substrate adhesion to four common extracellular matrix substrates was measured in the presence of either functional-blocking monoclonal antibodies, or with different divalent metal cations in solution. This study showed that melanocytes used only one or two different integrins to adhere to a particular extracellular matrix substrate, and that melanocytes derived from each tumour progression phase used the same integrins to adhere to a particular substrate. Furthermore, the presence of manganese cation in solution increases the degree of cell-substrate adhesion to all substrates, compared to calcium-magnesium containing media. Finally, manganese can increase the cell-substrate adhesion to all substrates by the RGP WM 35 melanoma cell line, and also increase the adhesion to basement-membrane specific substrates by the VGP melanoma cell lines.

MATERIALS AND METHODS:

Materials

Materials and culture media were obtained as described in Chapter III (p. 39). Monoclonal antibodies were obtained as described in Chapter IV (p. 81).

Cell Lines and Culture Conditions:

Cell lines and culture conditions were as described in Chapter III (p. 39).

Preparation of Extracellular Matrix Components

Preparation of substrates was as described in Chapter V (p. 105).

MTT-Microculture Tetrazolium Assay

Performance of the tetrazolium assay was as described in Chapter V (p. 106).

Centrifugation Cell Detachment Assay

The centrifugation assay was performed as described in Chapter V (p. 106).

Antibody Perturbation of Adhesion of Malignant Melanocytes to Extracellular Matrix Components

Cells were harvested and washed as described earlier. Selective blockade of integrin subunits was achieved by adding monoclonal antibodies against integrin subunits to the incubation medium which are known to block functional activity [α2, (P1E6); α3, (P1B5); α5, (P1D6); ß1, (4B4); and ß4 (3E1)]. Cell-substrate adhesion by melanocytes was examined using the standard assay conditions as described earlier, except cells were pretreated in serum-free media containing a 1/20 diluted antibody for 20 min, followed by a 1 hr incubation in the precoated microwells in serum-free media containing 1/100 diluted antibody.

Divalent Cation Perturbation Studies on Cell-Substrate Adhesion

Divalent cations were selectively added or deleted from the incubation media to determine the effect of these cations on cell adhesion. Normal melanocyte adhesion strength was measured on four substrates: laminin (10 µg/ml), fibronectin (10 µg/ml), collagen type I (10 µg/ml), and collagen type IV (10 µg/ml). Standard assay conditions were used as described earlier, except the cells were incubated on the substrate for 1 hr in Tris-saline (50 mM Tris; 150 mM NaCl); (pH 7.4) containing one of the following: 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, or 1 mM $MgCl_2$ and 1 mM $MnCl_2$.

RESULTS

Effect of anti-integrin antibodies on melanocyte adhesion

Perturbation of integrin function using monoclonal antibodies is determined by measuring the decrease in cell-substrate adhesion in the presence of monoclonal antibodies. Since the parameter of interest was the difference in adhesion, it would be easier to define differences between cells that had a high adhesion, rather than between cells that had a low adhesion. Many of the cell lines have low levels of adhesion (WM 35, WM 902B, WM 1341D, and SK-MEL 23). Antibody perturbation of cell-substrate adhesion was determined using only two cell lines, normal melanocytes and the metastatic growth phase melanoma, G 361. These cells were selected because they represent opposite ends of the differentiation spectrum, and from distant growth phases of tumour progression. Furthermore, the levels of adhesion to all substrates were sufficient to allow differences in cell-substrate adhesion to be detectable.

The degree of inhibition of cell-substrate adhesion for both cells by an anti-integrin antibody was dependent on the substrate (Figures VI-1 to VI-4). Adhesion to fibronectin by both normal melanocytes and G 361 could be blocked by antibodies against the α5-, and ß1-integrins. No perturbation of adhesion was observed with anti-α4, or anti-ß3 integrin antibodies, which could recognize alternative adhesion domains on fibronectin. The adhesion of normal melanocytes and G 361 were inhibited to the same degree by the anti-$\alpha 5$ antibody (30% of control), and to 40% of control by the anti-ß1 antibody. Thus, both the normal melanocytes and the metastatic melanoma cells, G 361 appear to use only the $\alpha 5ß1$ integrin to adhere to fibronectin.

Adhesion to laminin of both normal melanocytes and G 361 cells could be equally blocked by antibodies against $\alpha 2$ (50% of control), $\alpha 3$ (30% of control), and ß1 (6% of control). In addition, the anti-ß4 antibody perturbed adhesion to laminin by normal melanocytes (23% of control), but not G 361 (87% of control). One explanation could be the presence of a low level of ß4 integrin expression found on normal melanocytes (see Chapter III), which was not found on G 361 cells. Therefore, both normal melanocytes and G 361 cells use at least two integrins to adhere to laminin, including $\alpha 3ß1$, and $\alpha 2ß1$. Additionally, normal melanocytes use ß4-integrin (presumably as the $\alpha 6ß4$ integrin heterodimer) to adhere to laminin.

Adhesion to collagen Type I and Type IV by both normal and G 361 cells could be blocked by the antibodies against $\alpha 2$ (66% of control, and 57% of control, respectively), and ß1-integrins (5% of control, and 8% of control, respectively). The antibody against $\alpha 3$-integrin had minimal effect on adhesion (94% of control, and 90% of control, respectively). Thus, only the $\alpha 2ß1$ integrin appears to be responsible for adhesion of both the normal melanocytes and G 361 cells to collagen type I and IV.

These findings suggest that all melanocytes use the same integrin combinations for binding to extracellular matrix components. This would also imply that malignant transformation does not result in an alteration of integrin function, which was also found by Kaur and Carter [1992]. They looked at the expression and function of integrins expressed by a normal and virally-transformed epithelial cell line. Thus, while the substrate affinity of integrins can be cell-type specific [Gailit and Clark, 1993], the substrate affinity for the integrins expressed on normal melanocytes and G 361 melanoma cells in the metastatic growth phase appears to be similar. This may include the possibility that only these integrins are in a constitutively activated state, under these culture conditions. It could still be argued that integrin-substrate affinity may be altered by extraneous factors, which could either up-regulate or down-regulate cell-substrate adhesion. The observed variation in cell-substrate adhesion between cell lines, and the perceived lack of coordination between the degree of integrin expression and the strength of cell-substrate adhesion could be explained by this modulation of integrin affinity for the extracellular matrix substrate.

Effect of divalent cations on adhesive strength of melanocytes

Normal melanocytes adhered best to fibronectin, laminin, or Type I or IV collagens in the presence of Mn^{+2}, followed by (in descending order), Mg^{+2}/Ca^{+2}, Mg^{+2}/Mn^{+2}, Mg^{+2}, and Ca^{+2} (Table VI-1 to VI-4). Similarly, the VGP-WM 39 cell line adhered best to all substrates in the presence of Mn^{+2}/Mg^{+2}, followed by Mn^{+2}, Mg^{+2}/Ca^{+2}, Mg^{+2}, and Ca^{+2} (Table VI-1 to VI-4). Cell-substrate adhesion by normal and malignant melanocytes was sensitive to alterations in the cation species. The strength of cell-substrate adhesion could be greatly influenced by substituting one cation species for another. Furthermore, for a given cell line, this cation sensitivity was the same for each extracellular matrix substrate tested. All cell lines, regardless of tumour progression phase exhibited an increased substrate adhesion with manganese present, although the extent to which the adhesion was increased was different for each cell line (Figures VI-5 to VI-8). For example, the RGP WM 35 and the VGP cell lines had a statistically significant increase in adhesion to laminin in the presence of manganese. However, the normal melanocytes and the MGP cell lines had only a minimal increase in cell-substrate adhesion. Thus, the effect of manganese on cell-substrate adhesion was dependent in part on the cell line, the extracellular substrate, and perhaps also the phase of tumour progression.

In general, cells adhere best in Mn^{+2}-containing media, followed by Mg^{+2}-containing media, while Ca^{+2}-containing media provides the lowest level of cell-substrate adhesion. This dominance by manganese on cell-substrate adhesion strength between the different extracellular matrix substrates may imply an intrinsic property of integrins, and their interaction with the extracellular substrates. Indeed, this property might be exploited by cells in order to respond to changes in the extracellular environment, which could alter cell-substrate adhesive strength without the requirement for altering the surface expression of integrins.

Since the effect of cell-substrate adhesion by divalent cations may be sensitive to the state of cellular differentiation, cells were exposed to the differentiation-inducing agents, BRDU and α-MSH, and the cell-substrate adhesion was compared. The RGP WM 35 melanoma cell line greatly increased the degree of cell-substrate adhesion in the presence of manganese (Figure VI-9 to VI-12). Following exposure to BRDU, there was only a minimal increase in cell-substrate adhesion by all cell lines in the presence of manganese (Figure VI-9 to VI-12). This finding was also observed with the VGP WM 39 melanoma cell line, although the differences were seen only with the basement membrane-specific substrates laminin, and collagen type IV. No significant difference in cell-substrate adhesion could be seen between different states of cellular differentiation in the VGP and MGP melanoma cell lines. Therefore, the changes in cellular differentiation induced in WM 35 by BRDU increased the cell-substrate adhesion, which was minimally enhanced by the presence of manganese. Thus, changes in the state of differentiation within cells at particular phases of tumour progression may alter the integrin-mediated cell-substrate adhesion. This may suggest that the functional activation state of the integrins expressed by a cell type may be altered by changes in differentiation, and that this sensitivity may only be present at specific phases of tumour progression.

An unexpected observation was the significant increase in cell-substrate adhesion by all cells to blocked plastic in the presence of manganese-containing media (Figure VI-5). Upon further investigation, it was found that the adhesion to plastic was independent of the type of blocking agent used, but dependent on the manganese concentration (see Appendix B). Adhesion could be blocked in a concentration-dependent manner by soluble GRGDS peptide, but not by the control peptide, GRGES, and by perturbation by anti-α3 and ß1-integrin antibodies, implying that integrins were involved at some point in the adhesion process. Alternatively, the presence of manganese may change the signal transduction pathways within the cells, improving the formation of an adhesion complex.

Therefore, the generalized increase in cell-substrate adhesion observed in the presence of manganese might be due to a non-specific activation of integrins through a conformational change brought on by the occupation of the divalent cation-binding site by manganese. This conformational change is perceived by the cell to be a receptor that has been bound to its ligand, which causes a series of signals within the cell that leads to increased adhesion. Alternatively, the change in the integrin conformation induced by manganese may recognize a partial adhesion sequence on the blocking substrate. Thus, integrins are a necessary, but not complete requirement for this adhesion increase, since it can be perturbed by both functional blocking antibodies, and soluble GRGDS peptide. However, the nature of this signal, and what the message is to the cell, are not known.

DISCUSSION

Normal and malignant melanocytes use the same integrin subunits to adhere to extracellular matrix substrates.

This study has shown that both normal and malignant melanocytes use only a limited number of integrins to adhere to extracellular matrix substrates. Furthermore, both normal and malignant melanocytes use the α5β1 integrin to adhere to fibronectin, the α2β1 integrin to adhere to collagen Type I and IV, and both the α2β1 and α3β1 integrins to adhere to laminin. In addition, normal melanocytes appear to use the α6β4 integrin to assist adhesion to laminin. Thus, melanocytes in all phases of tumour progression utilize the same integrin subunits for adhesion to extracellular matrix substrates, and this data does not support a role for novel integrin subunits being responsible for cell-substrate adhesion during tumour progression. As these integrins are also the ones most highly expressed by malignant melanocytes at all stages of tumour progression, it is reasonable to expect that modulation of the function of these integrin subunits would have significant effects on cell-substrate adhesion to extracellular matrix substrates.

Studies by investigators using other cell systems have also demonstrated a constancy in integrin function between normal and transformed cell lines. Kaur and Carter [1992] examined integrin expression and function in normal keratinocytes which were also virally transformed to squamous cell carcinoma. They found that both the normal and transformed cell lines expressed the same integrin subunits, and that the expressed integrins had equivalent functional adhesion properties in their assay. They concluded that the transformation of the epidermal cells did not result in the alteration in the function of the integrins [Kaur and Carter, 1992]. While a particular integrin subunit expressed by different cell types may exhibit different affinities for extracellular matrix substrates, it appears that integrins expressed within the same cell type adhere to the same extracellular matrix substrates.

Divalent cations can increase integrin-mediated adhesion by conformational activation

This study also found that cell-substrate adhesion by normal and malignant melanocytes was sensitive to alterations in the extracellular divalent metal cation species, with manganese providing the best cell-substrate adhesion, followed by magnesium, then calcium. Grzesiak and colleagues [1992] found that different divalent metal cation species are capable of occupying the metal ion binding domains in the α-integrin subunit, modulating the adhesion capability of integrins. They suggest that occupancy of the metal ion binding domain changes the conformational shape of the integrin, which would then open a ligand-binding domain. The hypothesis was put forth by Grzesiak and colleagues [1992] and Humphries [1993] who implied that different metal ions have specific ionic radii, which can fit within the coordination binding site on the α-integrin subunit. Since the ionic radii of the metal ions are different, the binding site will be either compressed or expanded depending on the ionic radius. This compression or expansion of a critical domain on the integrin subunit may produce a conformational change which is transmitted along the length of the molecule into the cytoplasmic domain. Such an alteration may then lead to a different set of signals within the cell, which is ultimately translated into changed cell behaviour.

Substitution of manganese as the extracellular cation within the cation-binding site on the α-integrin subunit appears to activate the adhesion function of integrins expressed by cells. This is in concordance with the findings of Grinnell and colleagues [1991], who found that manganese could cause the integrins to produce an activated conformation. Their study produced a monoclonal antibody that recognized only integrins which could bind to its substrate. Integrins which did not bind to the substrate were not recognized by this antibody. They found that integrins which were nonadherent could be made adherent in the presence of manganese, and the M16 antibody recognized its epitope on the manganese-treated integrins. Thus, manganese appears to be able to produce a conformational change in the integrin structure which permits adhesion.

Since cells of the same lineage expressed the same integrins capable of binding to extracellular matrices, it was surprising to discover that cell lines at different phases of tumour progression were unable to adhere to extracellular matrices, despite their expression of equivalent levels of surface integrins. In particular, the RGP WM 35 cell line had poor adhesion to all substrates, despite having the highest levels of integrin expression (see Chapter IV). Furthermore, the VGP melanoma cell lines had poor adhesion to laminin and collagen type IV, despite substantial expression of α2β1 and α3β1 integrin. Perhaps the expressed integrins by these cells were nonfunctional. The restoration of adhesion for all cell lines with manganese implies that the integrins could be made functional, but were inactivated in some way in the native state. This selective inactivation of integrins by RGP (all substrates) and VGP cell lines (laminin and collagen type IV) is probably an important step in tumour progress, for it could explain what is observed *in situ*. Melanoma cells in RGP lose attachment to the basement membrane and can be located in the epidermis. Furthermore, melanoma cells in VGP must be capable of leaving the epidermal compartment containing laminin and collagen type IV, and migrate into the dermis, which is rich in fibronectin and collagen type I. Selective inactivation of integrins by melanoma cells would reduce adhesion to these substrates, and permit cells to migrate more freely, without being bound to the substrate. If this is indeed the case, it could be important to examine how these cells can alter this activation of integrin function. Currently, these mechanisms are not well understood.

The restoration of adhesion by WM 35 following BRDU exposure might imply that the change in cell differentiation could activate integrin function. However, since BRDU has a wide variety of effects on protein methylation, in addition to the DNA effects (see Chapter III), any definitive explanation would be difficult. However, it illustrates how WM 35 may be a useful cell line for studying integrin activation, and how reversible changes in cell differentiation may be used to control function.

While manganese is not usually found in millimolar concentrations in physiological conditions, it does illustrate how an extracellular parameter may influence a specialized activity such as adhesion. Cells may be able to use local changes in the extracellular divalent cation concentration to modulate the integrin adhesion and interacellular signalling, without having to change the density of integrins expressed on the cell surface. One example of an environment with an altered extracellular cation concentration is during wound healing. The intracellular concentrations of magnesium and calcium are far in excess of the extracellular concentrations. Cell injury or death would cause the intracellular contents to be released into the extracellular environment. Adjacent cells would be exposed to these higher concentrations of cations, and may alter their integrin-mediated cell adhesion and migration in response [Zambruno et al, 1993]. Subsequently, when the wound is healed, and the local environment is re-established, the extracellular cation concentrations would return to prewounding levels, and the original integrin function would also return to normal.

Table VI-1: Effect of Divalent Cations on Cell-Substrate Adhesion on Fibronectin (Corrected Percent Adhesion)

Cation	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
Calcium	7.2 ± 1.0	0.0 ± 0.7	48.8 ± 4.2	10.3 ± 0.6	3.0 ± 1.5	0.0 ± 1.0	4.3 ± 2.0	7.5 ± 5.0
Magnesium	21.5 ± 2.5	0.0 ± 0.8	49.8 ± 3.1	13.7 ± 3.0	14.5 ± 3.0	4.1 ± 3.0	31.3 ± 2.5	7.2 ± 1.8
Manganese	56.1 ± 1.5	78.2 ± 4.3	81.1 ± 2.3	24.2 ± 9.9	72.2 ± 5.9	12.8 ± 3.0	51.3 ± 1.9	33.8 ± 2.0
Mn/Mg	25.9 ± 2.5	67.8 ± 4.1	83.1 ± 2.0	57.5 ± 8.9	87.4 ± 5.2	31.6 ± 3.0	49.1 ± 1.5	25.8 ± 2.0
Mg/Ca	51.7 ± 2.5	0.0 ± 0.1	53.9 ± 4.5	17.3 ± 4.9	30.0 ± 3.7	7.3 ± 1.7	34.8 ± 1.7	0.0 ± 2.0

Corrected Percent Adhesion = (Percent Adhesion with cation present) - (Percent adhesion with EDTA (10 mM) present) ± standard deviation

Percent Adhesion with EDTA present: NHEM, 2.0; WM35, 6.7; WM39, 16.9; WM902B, 33.7; WM1641D, 1.7; SK23, 5.0; G361, 3.8; C32, 13.7.

N = Normal
NHEM = Normal human epidermal melanocytes
RGP = Radial growth phase
VGP = Vertical growth phase
Mn = Manganese
Mg = Magnesium
Ca = Calcium

Table VI-2: Effect of Divalent Cations on Cell-Substrate Adhesion on Laminin (Corrected Percent Adhesion)

Cation	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
Calcium	2.2 ± 1.0	0.0 ± 0.7	7.7 ± 5.0	3.3 ± 3.0	1.2 ± 0.3	5.8 ± 3.0	31.8 ± 1.5	7.9 ± 5.0
Magnesium	21.7 ± 2.0	11.7 ± 5.8	13.3 ± 1.6	2.0 ± 2.3	1.2 ± 0.6	20.9 ± 3.0	32.7 ± 2.7	3.5 ± 1.3
Manganese	64.1 ± 1.5	34.7 ± 5.0	53.4 ± 4.0	25.8 ± 5.7	17.2 ± 3.2	24.6 ± 2.0	47.8 ± 0.6	24.9 ± 1.7
Mn/Mg	44.6 ± 2.5	36.2 ± 3.8	71.7 ± 1.4	65.0 ± 7.8	21.3 ± 3.2	29.3 ± 2.8	46.6 ± 1.0	11.1 ± 3.3
Mg/Ca	47.1 ± 3.0	0.0 ± 0.2	25.3 ± 4.5	0.5 ± 0.8	4.0 ± 1.2	18.9 ± 4.0	36.7 ± 2.7	0.0 ± 0.8

Corrected Percent Adhesion = (Percent Adhesion with cation present) - (Percent adhesion with EDTA (10 mM) present) ± standard deviation

Percent Adhesion with EDTA present: NHEM, 3.5; WM35, 5.7; WM39, 7.7; WM902B, 13.5; WM1641D, 0.3; SK23, 1.3; G361, 6.9; C32, 7.1

N = Normal
NHEM = Normal human epidermal melanocytes
RGP = Radial growth phase
VGP = Vertical growth phase
Mn = Manganese
Mg = Magnesium
Ca = Calcium

Table VI-3: Effect of Divalent Cations on Cell-Substrate Adhesion on Collagen Type I (Corrected Percent Adhesion)

Cation	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
Calcium	1.0 ± 1.2	0.0 ± 1.3	0.0 ± 3.1	7.0 ± 2.2	2.2 ± 1.3	3.1 ± 2.0	0.0 ± 2.0	11.1 ± 7.0
Magnesium	28.2 ± 2.0	1.1 ± 0.4	31.1 ± 2.6	15.6 ± 6.9	0.0 ± 0.7	12.4 ± 2.0	39.8 ± 0.9	4.7 ± 2.0
Manganese	57.4 ± 2.0	28.5 ± 3.1	66.6 ± 4.4	20.9 ± 6.3	22.2 ± 2.0	17.5 ± 4.0	52.3 ± 1.3	22.7 ± 4.0
Mn/Mg	35.5 ± 3.0	26.5 ± 2.8	76.1 ± 8.9	60.0 ± 6.5	21.3 ± 8.2	32.2 ± 5.0	46.5 ± 1.7	16.4 ± 0.4
Mg/Ca	41.9 ± 3.0	0.0 ± 0.6	38.8 ± 8.8	8.7 ± 2.7	3.9 ± 1.6	14.0 ± 4.0	44.5 ± 1.2	0.0 ± 3.0

Corrected Percent Adhesion = (Percent Adhesion with cation present) - (Percent adhesion with EDTA (10 mM) present) ± standard deviation

Percent Adhesion with EDTA present: NHEM, 2.0; WM35, 4.4; WM39, 14.3; WM902B, 21.3; WM1641D, 0.0 SK23, 0.7; G361, 3.9; C32, 7.7

N = Normal
NHEM = Normal human epidermal melanocytes
RGP = Radial growth phase
VGP = Vertical growth phase
Mn = Manganese
Mg = Magnesium
Ca = Calcium

Table VI-4: Effect of Divalent Cations on Cell-Substrate Adhesion on Collagen Type IV (Corrected Percent Adhesion)

Cation	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
Calcium	3.9 ± 0.6	0.3 ± 0.4	1.9 ± 2.8	0.0 ± 3.0	2.9 ± 0.5	5.2 ± 3.0	3.0 ± 1.3	1.2 ± 1.6
Magnesium	32.1 ± 3.0	4.9 ± 0.8	33.2 ± 3.6	5.7 ± 2.8	1.3 ± 0.5	21.0 ± 1.5	35.5 ± 2.4	6.3 ± 2.5
Manganese	77.7 ± 3.0	18.6 ± 2.5	58.2 ± 3.5	1.3 ± 1.5	24.4 ± 2.2	20.7 ± 3.0	52.2 ± 2.1	15.7 ± 3.0
Mn/Mg	43.2 ± 2.5	20.6 ± 3.9	77.2 ± 2.2	59.5 ± 9.7	17.5 ± 4.2	31.5 ± 4.0	48.5 ± 2.0	15.2 ± 4.0
Mg/Ca	47.1 ± 2.0	2.9 ± 0.7	47.0 ± 4.8	11.8 ± 3.4	6.1 ± 0.8	22.1 ± 3.5	41.5 ± 1.9	0.3 ± 0.6

Corrected Percent Adhesion = (Percent Adhesion with cation present) - (Percent adhesion with EDTA (10 mM) present) ± standard deviation

Percent Adhesion with EDTA present: NHEM, 1.5; WM35, 3.7; WM39, 10.2; WM902B, 29.2; WM1641D, 0.0; SK23, 1.3; G361, 2.5; C32, 6.0

N = Normal
NHEM = Normal human epidermal melanocytes
RGP = Radial growth phase
VGP = Vertical growth phase
Mn = Manganese
Mg = Magnesium
Ca = Calcium

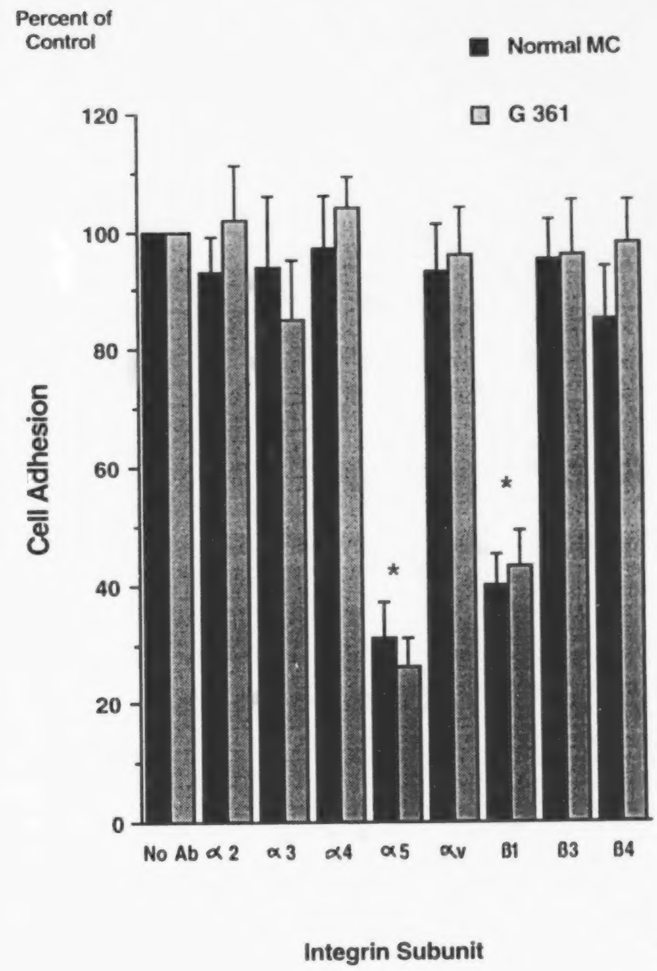
Table VI-5: Effect of Divalent Cations on Cell-Substrate Adhesion on Milk Powder Blocked Plastic
(Corrected Adhesion)

Cation	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
Calcium	1.5 ± 0.8	0.0 ± 0.8	0.0 ± 0.8	0.0 ± 3.1	0.3 ± 1.9	0.0 ± 1.0	0.4 ± 0.5	0.0 ± 1.0
Magnesium	6.9 ± 1.3	0.0 ± 0.6	0.0 ± 0.8	0.0 ± 2.3	0.0 ± 0.5	0.0 ± 1.0	2.1 ± 0.8	0.0 3.0
Manganese	22.5 ± 0.5	9.8 ± 3.6	37.4 ± 9.1	8.9 ± 0.0	6.9 ± 3.0	13.8 ± 3.0	51.9 ± 0.6	2.5 ± 3.0
Mn/Mg	4.6 ± 1.5	21.5 ± 4.6	68.3 ± 4.7	43.1 ± 2.6	6.3 ± 3.4	14.9 ± 3.0	48.6 ± 1.2	0.0 ± 3.0
Mg/Ca	5.9 ± 1.0	0.0 ± 0.4	3.1 ± 2.8	0.0 ± 1.4	0.0 ± 2.1	7.2 ± 6.0	24.7 ± 2.9	0.0 ± 2.0

Corrected Percent Adhesion = (Percent Adhesion with cation present) - (Percent adhesion with EDTA (10 mM) present) ± standard deviation

Percent Adhesion with EDTA present: NHEM, 2.0; WM35, 6.7; WM39, 16.9; WM902B, 33.7; WM1641D, 1.7; SK23, 5.0; G361, 3.8; C32, 13.7.

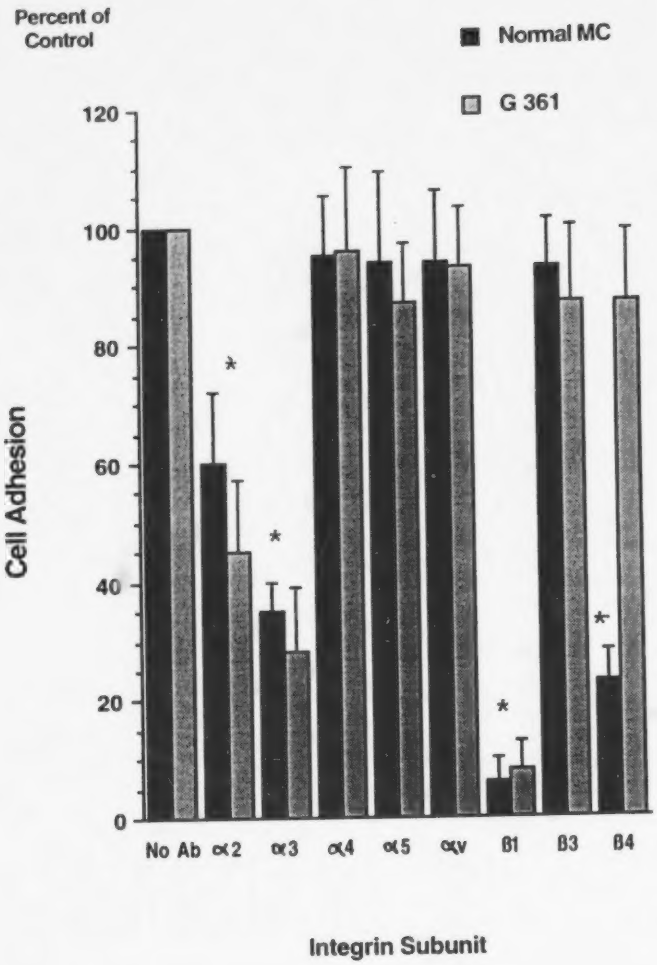
N = Normal
NHEM = Normal human epidermal melanocytes
RGP = Radial growth phase
VGP = Vertical growth phase
Mn = Manganese
Mg = Magnesium
Ca = Calcium



* $p < 0.05$

FIGURE VI-1: Effect of Blocking Antibodies on Cell-Substrate Adhesion to Fibronectin by Normal Melanocytes and G 361 Melanoma Cells

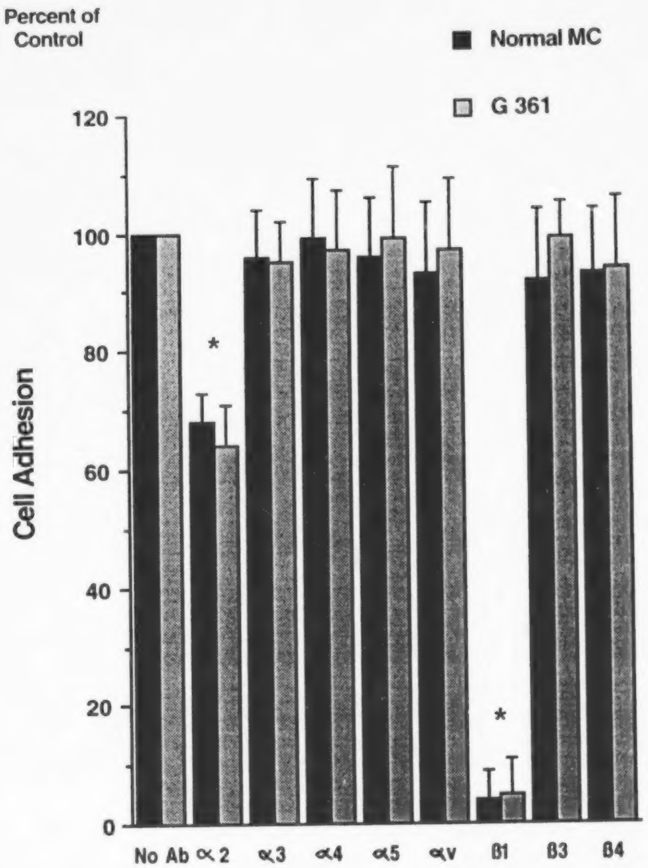
Antibodies against α5 and ß1-integrins had the greatest effect on cell adhesion strength to this substrate for both normal melanocytes and G 361 melanoma cells.



* $p < 0.05$

FIGURE VI-2: Effect of Blocking Antibodies on Cell-Substrate Adhesion to Laminin by Normal Melanocytes and G 361 Melanoma Cells

Antibodies against α2, α3 and ß1-integrins had the greatest effect on cell adhesion strength to this substrate for both normal melanocytes and G 361 melanoma cells, and ß4-integrin interfered with normal melanocyte adhesion strength.

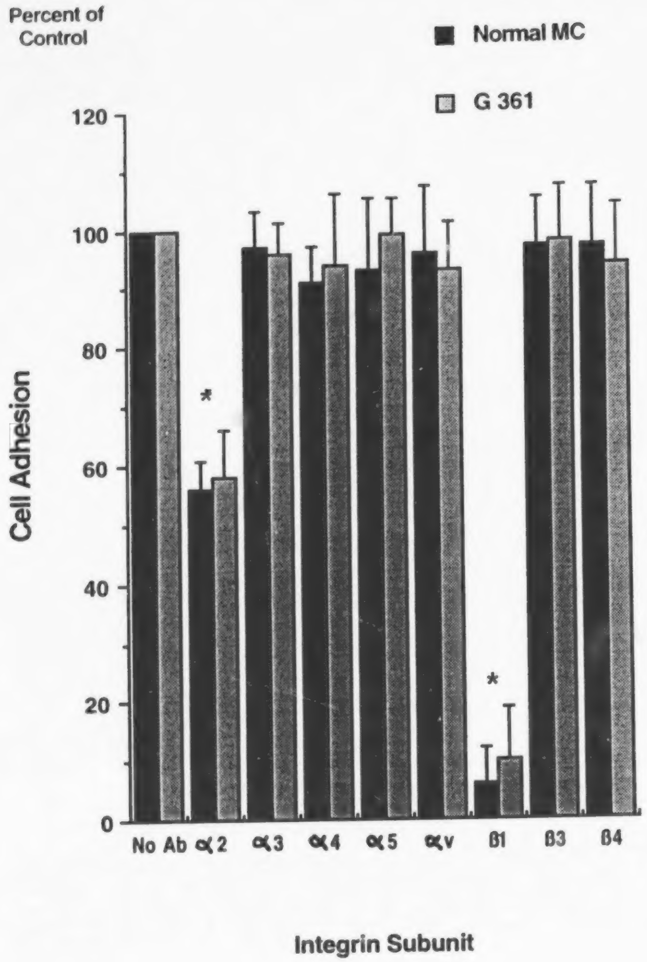


Integrin Subunit

* $p < 0.05$

FIGURE VI-3: Effect of Blocking Antibodies on Cell-Substrate Adhesion to Collagen Type I by Normal Melanocytes and G 361 Melanoma Cells

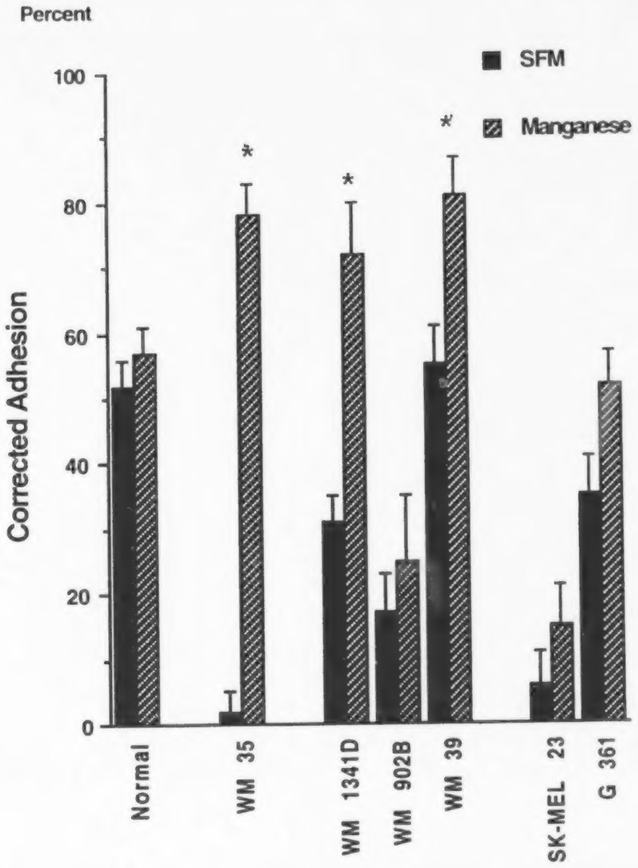
Antibodies against α2 and ß1-integrins had the greatest effect on cell adhesion strength to this substrate for both normal melanocytes and G 361 melanoma cells.



* $p < 0.05$

FIGURE VI-4: Effect of Blocking Antibodies on Cell-Substrate Adhesion to Collagen Type IV by Normal Melanocytes and G 361 Melanoma Cells

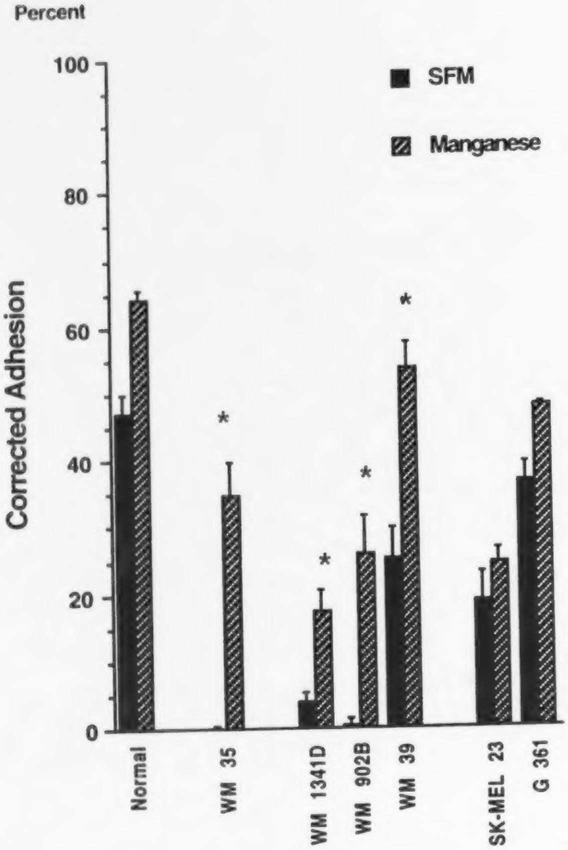
Antibodies against α2 and β1-integrins had the greatest effect on cell adhesion strength to this substrate for both normal melanocytes and G 361 melanoma cells.



* $p < 0.05$

FIGURE VI-5: Effect of Manganese on Adhesion to Fibronectin by Normal and Malignant Melanocytes

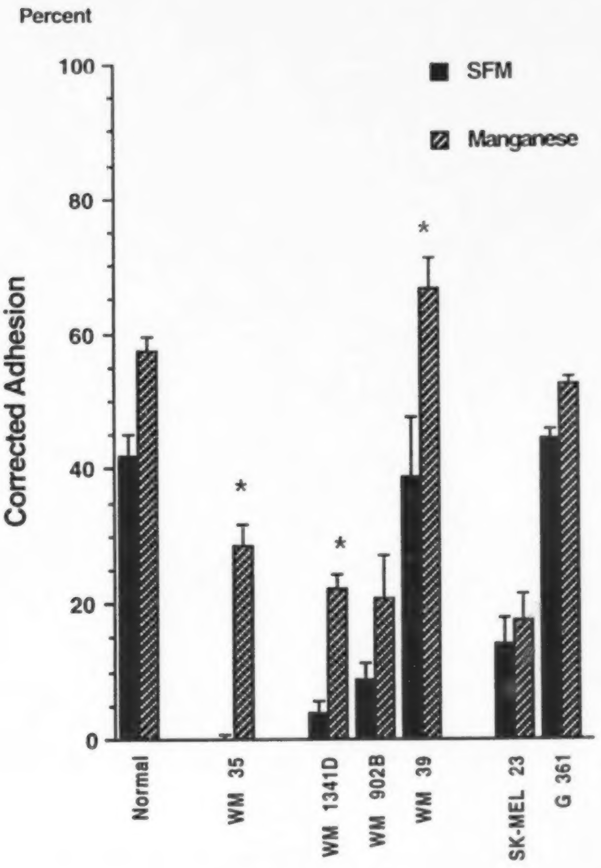
Manganese (1 mM) increased adhesion strength to this substrate compared to serum free media (SFM) for WM 35, and WM 1341D cells, but had a minimal increase with all other cells. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE VI-6: Effect of Manganese on Adhesion to Laminin by Normal and Malignant Melanocytes

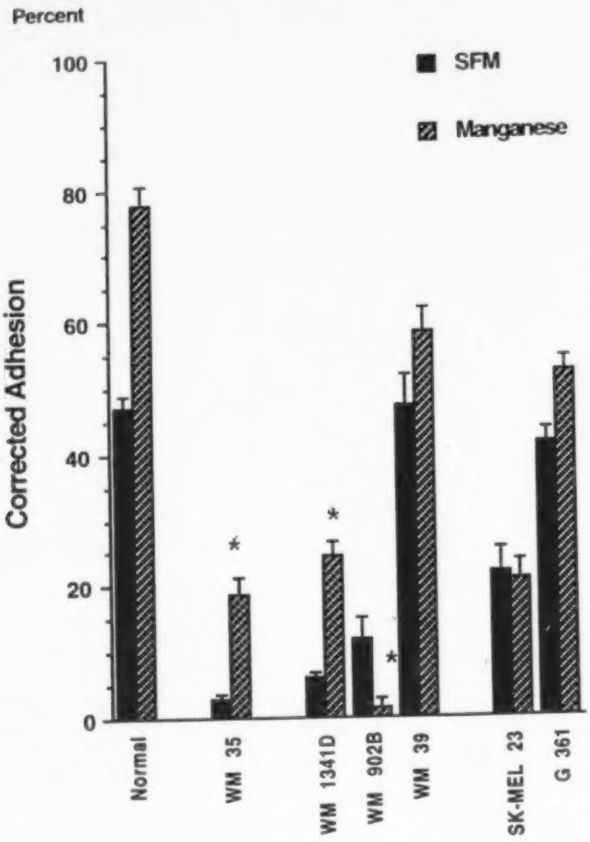
Manganese (1 mM) increased adhesion strength to this substrate compared to serum free media (SFM) for WM 35, and the VGP cells, but had a minimal increase with all other cells. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE VI-7: Effect of Manganese on Adhesion to Collagen Type I by Normal and Malignant Melanocytes

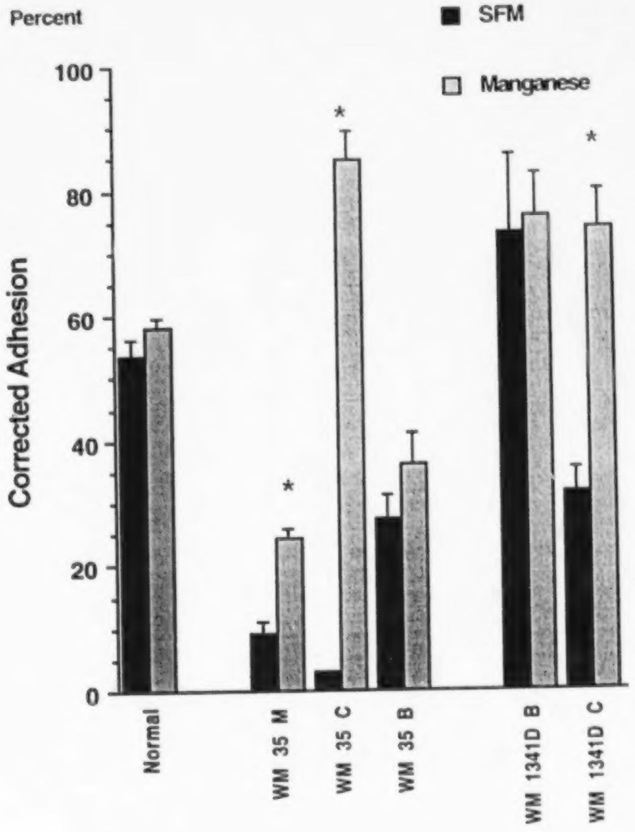
Manganese (1 mM) increased adhesion strength to this substrate compared to serum free media (SFM) for WM 35, and WM 1341D cells, but had a minimal increase with all other cells. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE VI-8: Effect of Manganese on Adhesion to Collagen Type IV by Normal and Malignant Melanocytes

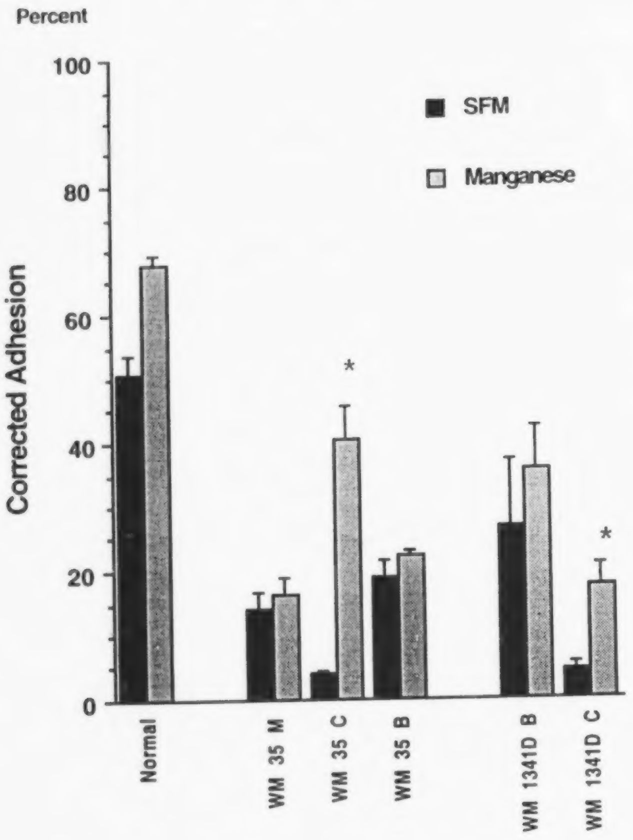
Manganese (1 mM) increased adhesion strength to this substrate compared to serum free media (SFM) for WM 35, and WM 1341D cells, but had a minimal increase with all other cells. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic.



* $p < 0.05$

FIGURE VI-9: Effect of BRDU and α-MSH on Adhesion to Fibronectin in the Presence of Manganese by Normal and Malignant Melanocytes

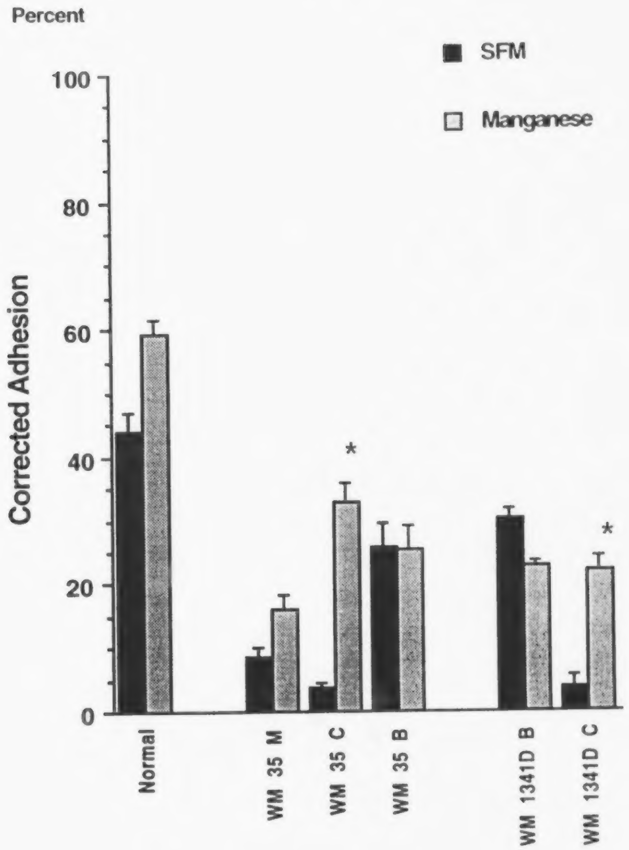
BRDU-exposed cells (B) and α-MSH-exposed cells (M) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. Relative to the unexposed control cells, exposure to differentiation-induction agents minimized the increase in cell-substrate adhesion strength produced by 1 mM manganese. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic. SFM = serum free media.



* $p < 0.05$

FIGURE VI-10: Effect of BRDU and α-MSH on Adhesion to Laminin in the Presence of Manganese by Normal and Malignant Melanocytes

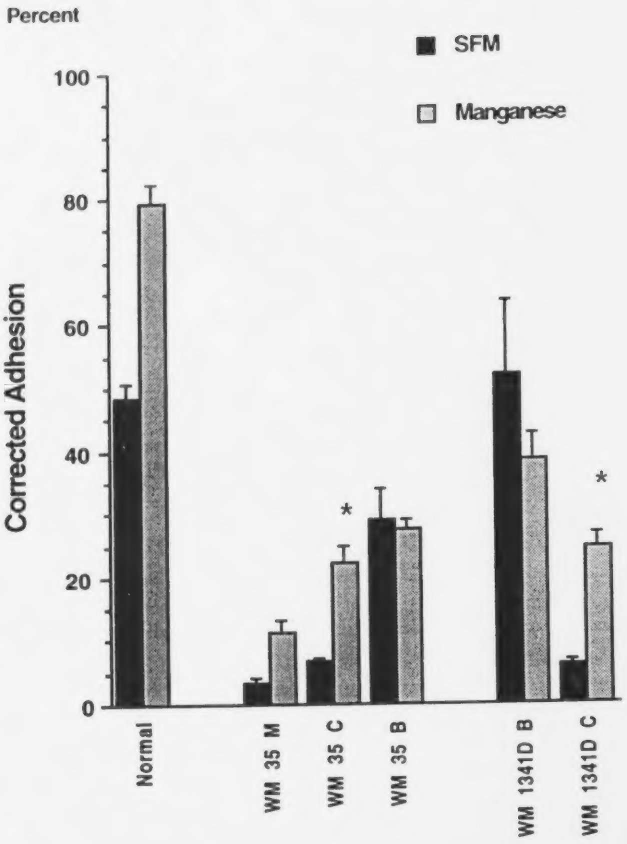
BRDU-exposed cells (B) and α-MSH-exposed cells (M) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. Relative to the unexposed control cells, exposure to differentiation-induction agents minimized the increase in cell-substrate adhesion strength produced by 1 mM manganese. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic. SFM = serum free media.



* $p < 0.05$

FIGURE VI-11: Effect of BRDU and α-MSH on Adhesion to Collagen Type I in the Presence of Manganese by Normal and Malignant Melanocytes

BRDU-exposed cells (B) and α-MSH-exposed cells (M) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. Relative to the unexposed control cells, exposure to differentiation-induction agents minimized the increase in cell-substrate adhesion strength produced by 1 mM manganese. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic. SFM = serum free media.



* $p < 0.05$

FIGURE VI-12: Effect of BRDU and α-MSH on Adhesion to Collagen Type IV in the Presence of Manganese by Normal and Malignant Melanocytes

BRDU-exposed cells (B) and α-MSH-exposed cells (M) are positioned relative to their unexposed control cells (C) according to the effect upon cellular differentiation, such that more differentiated cells are to the left, and less differentiated cells are to the right. Relative to the unexposed control cells, exposure to differentiation-induction agents minimized the increase in cell-substrate adhesion strength produced by 1 mM manganese. Corrected adhesion is the difference between the experimental adhesion strength and adhesion strength to blocked plastic. SFM = serum free media.

BIBLIOGRAPHY

Eisinger M, Marko O. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc Natl Acad Sci USA 79:2018-2022, 1982.

Gailit JO, RAF Clark. Integrins. Adv.Dermatol. 8:129-153, 1993.

Grzesiak JJ, GE Davis, D Kirchhofer, MD Pierschbacher. Regulation of $\alpha 2\beta 1$-mediated fibroblast migration on type I collagen by shifts in the concentrations of extracellular Mg^{+2} and Ca^{+2}. J.Cell Biol. 117:1109-1117, 1992.

Grinnell F, R Backman. Role of integrin receptors in manganese-dependent BHK cell spreading on albumin-coated substrata. Exp.Cell Res 195:218-223, 1991.

Halaban R, FD Alfano. Selective elimination of fibroblasts from cultures of normal human melanocytes. In Vitro 20:447-450, 1984.

Humphries MJ, AP Mould, DS Tuckwell. Dynamic aspects of adhesion receptor function - integrins both twist and shout. BioEssays 15:391-397, 1993.

Kaur P, WG Carter. Integrin expression and differentiation in transformed human epidermal cells is regulated by fibroblasts. J.Cell Sci. 103:755-763, 1992.

Lotz MM, CA Burdsal, HP Erickson, DR McClay. Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. J.Cell Biol, 109:1795-1805, 1989.

Zambruno G, PC Marchisio, A Melchiori, S Bondanza, R Cancedda, M DeLuca. Expression of integrin receptors and their role in adhesion, spreading and migration of normal human melanocytes. J. Cell Sci. 105:179-190, 1993.

VII. SUMMARY AND DISCUSSION

A. SUMMARY

In this thesis I have proposed six major hypotheses to be tested. Human melanocytes possess several phases of tumour progression which are clinically and histopathologically distinct. These include (a) radial growth phase (RGP), (b) vertical growth phase (VGP), and (c) metastatic growth phase (MGP). I have hypothesized that the cells in each phase of tumour progression will express:

1. fewer markers of cellular differentiation than that in the preceding phase;

2. different integrin subunits on their cell surface from that of the preceding phase; and

3. binding to extracellular matrix substrates with an adhesion strength different from the preceding phase.

The expression of the above three may be altered chemically by using differentiation-inducing agents, and therefore the cells in each phase of tumour progression will change:

4. the expression of cellular differentiation markers in a pattern consistent with either increasing or decreasing differentiation;

5. the expression of cell surface integrin in a pattern similar to that expressed by the adjacent tumour progression phase closest in the direction of alteration of differentiation; and

6. the adhesion strength to extracellular matrix substrates in a pattern similar to that exhibited by the adjacent tumour expression phase closest in the direction of alteration of cellular differentiation.

These hypotheses are verified as follows:

Hypothesis 1: Cell Differentiation and Growth Phases

Comparisons of the expression of cellular differentiation markers (i.e., cell morphology, melanosomal structure, tyrosinase activity, and melanin content), and cellular growth indicators (i.e., doubling times, soft agar colony formation, and cell migration rates) have clearly shown that malignant melanocytes are less differentiatied and have greater growth potentials than normal melanocytes (Chapter III). Furthermore, the degree of differentiation decreased with advancing phases of tumour progression. Thus, these findings support the view that tumour progression is a state of progressive loss of differentiation.

Hypothesis 2: Integrin Expression and Growth Phases

Human melanocytes express a limited repertoire of integrin subunits, and melanocytes expressed the same major integrin subunits in all phases of tumour progression (Chapter IV). However, there are differences in the degree of expression. For the major integrin subunits expressed by melanocytes ($\alpha2$, $\alpha3$, and $\beta1$), the levels of expression are greatest in the RGP, and the expression levels decline with more advanced phases of tumour progression.

Hypothesis 3: Adhesion Strength to Extracellular Matrix Substrates and Growth Phase

Normal human melanocytes adhered best to fibronectin, followed by laminin and the collagens (Chapter V). In contrast to expectation, the adhesion strength to all substrates was greatly diminished for the RGP cells, but was regained by the VGP cells. Compared to the VGP cells, the MGP cell lines had a lower strength of adhesion to all the substrates tested. The decreased adhesion strength observed with the RGP cells occurred despite having the highest expression levels of the major integrin subunits. However, the decrease in the adhesion strength by the VGP and MGP cells corresponded to the decrease in integrin subunit expression levels. Thus, the decrease in adhesion strength observed with the RGP cells could not be directly attributed to the level of integrin subunit expression. However, RGP cell-substrate adhesion strengths could be increased by adding manganese into the culture medium. Since manganese is thought to stabilize an activated conformational shape of the integrin molecule (See Chapter VI for discussion), it is possible that the integrins expressed by RGP cells are functionally inactivated, but can be made functionally active by manganese. Also, it appeared that adhesion to laminin by VGP cells was functionally inactivated, since the adhesion strength to laminin could be greatly increased by manganese (Chapter VI).

Hypothesis 4: Alteration of Cell Differentiation and Differentiation Marker Expression

The two differentiation-inducing agents, BRDU and α-MSH were able to alter the cellular differentiation phenotype of cells of malignant cell lines, but did not alter the phenotype of normal human melanocytes. In general, BRDU induced each malignant cell line to express markers with a less differentiated cellular phenotype (Chapter III). The only exception to this trend was the early VGP cell line, WM 1341D, which expressed a more differentiated cellular phenotype following BRDU exposure. Conversely, α-MSH induced cells to express a more differentiated cellular phenotype. Also, the degree of alteration induced by BRDU and α-MSH was greatest with the early RGP and VGP melanoma cells, whereas the more advanced MGP cells were much less responsive to the induction. Thus, the state of cellular differentiation could be reversibly altered by using either BRDU or α-MSH. These compounds appeared to have opposite effects on cellular differentiation, and the earlier growth phase cell lines were more responsive than the later growth phase cell lines.

Hypothesis 5: Alteration of Cell Differentation and Integrin Subunit Expression

The level of expression of major integrin subunits increased during the transition from normal to the RGP, whereas this level decreased during the progression through the VGP to the MGP metastatic growth phases (Chapter IV). The integrin expression levels of the cells exposed to the differentiation-inducing agents were more closely matched to those of the non-exposed cells in the adjacent tumour progression phase. For example, VGP cells which were exposed to BRDU became less differentiated and expressed the level of integrins similar to the non-exposed MGP cells. These findings suggest that not only do cells become less differentiated with tumour progression, but also the level of integrin subunit expression is linked to the differentiation state.

Hypothesis 6: Alteration of Cell Differentiation and Adhesion Strength

Cell-substrate adhesion strength was dependent upon the phase of tumour progression (Chapter V). Furthermore, the adhesion strength was also dependent upon the state of differentiation. Cells which became less differentiated following exposure to BRDU showed a cell-substrate adhesion strength more similar to that of the following tumour progression phase. Conversely, cells which became more differentiated had an adhesion strength that was more similar to that of the previous tumour progression phase . This included the decrease in cell-substrate adhesion observed for the RGP melanoma cells. These findings indicate that cell-substrate adhesion strength is also linked to the differentiation state.

B. CONCLUSION AND SIGNIFICANCE

This study appears to be the first one which has characterized the cellular and subcellular features unique to the human malignant melanocyte lines derived from different phases of tumour progression. While some features of the MGP cells used in the study are well known (SK-MEL 23 and G 361), the phenotypic characteristics of the RGP, and VGP cells of the present study have been previously unknown. Furthermore, this study also appears to be the first to examine and document the changes in differentiation state following exposure to the reversible differentiation-induction agents, BRDU, and α-MSH. It was found that melanoma cells in the early growth phase are more responsive to the effects of BRDU and α-MSH than the metastatic melanoma cells, and that multiple parameters which are used to determine the differentiation state had all changes in the same direction. Finally, this study is the first to characterize the *in vitro* expression of integrin subunits on these cells and also to determine the cell-substrate adhesion strength of these cell lines, in the presence and absence of differentiation-induction agents. Therefore, this study appears to be the first to attempt to examine integrin expression and function in relation to cellular differentiation and malignant tumour progression by using two different methods, i.e., human malignant melanocytes representing the natural tumour progression and those melanocytes exposed to the artificial differentiation-induction agents.

The findings support the idea that integrin expression is indeed linked to the state of differentiation, be it defined by the phase of tumour progression, or as a result of induction by chemical agents. Also, integrin function is linked to differentiation state, although not in a linear fashion.

The loss of functional activity exhibited by integrins expressed on RGP cells belies the complexity that is cell adhesion. Other regulatory mechanisms must be active in this phase, which can inactivate integrin function, since integrin function can be recovered by using an extracellular cation to stabilize an activated conformation. While none of these mechanisms of inactivation are known, it would be an area of great interest for future study, because of its importance in regulating cell attachment and migration during normal physiological processes such as embryonic migration and tissue patterning, and in abnormal processes such as malignant tumour metastasis. Potential avenues of approaching this area would be the identification of potential signalling molecules which co-localize with integrins on the cell surface, or changes in intracellular signal transduction pathways.

The findings of this study are clinically significant in three ways. First, by using cell lines derived from established phases of tumour progression, and by using differentiation-induction agents, we have confirmed the notion that malignant progression involves the progressive loss of differentiation. Thus, any attempts at halting or slowing this loss in differentiation could prevent or delay the appearance of malignancy. Most of the recent significant advances in cancer therapy have been directed at controlling the differentiation state of tumour cells [Kraemer et al, 1988].

Second, our findings indicate that integrin expression changes with tumour progression, albeit in a complex fashion. As suggested by Albelda and his colleagues [1990], we concur that while no single integrin subunit can distinguish between the different phases of tumour progression, perhaps a panel of several integrins may provide more diagnostic accuracy. This may provide a basis in establishing a better prognostic index for thin melanomas with a low risk for metastasis, since the progression from RGP to VGP has significant effects on the probability for metastatic disease.

Third, our findings of a loss of integrin function in RGP, and the re-acquisition of function in VGP may offer a potential avenue for therapy. A better understanding of the mechanisms responsible for this phenomenon may be exploited clinically, for if VGP cells can be reverted to a cell with RGP characteristics, the tumour progression might be aborted. Practical avenues of application may be through biological response modifiers which modulate the signal transduction pathways, or by antisense oligodeoxynucleotides directed at the signalling mechanisms.

In this study, the link between tumour progression and differentiation state has been established (Chapter III). Furthermore, the expression and function of integrins also tend to be linked to the phase of tumour progression and differentiation state. Thus, it can be concluded that integrins may play a critical role in the biology of tumour progression and the regulation of differentiation.

BIBLIOGRAPHY

Albelda SM, SA Mette, DE Elder, R Stewart, L Damjanovich, M Herlyn, C Buck. Integrin distribution in malignant melanoma: association of the β3 subunit with tumor progression. Cancer Res. 50:6757-64, 1990.

Kraemer KH, JJ DiGiovanna, AN Moshell, RE Tarone, GL Peck. Prevention of skin cancer in xeroderma pigmentosum with the use of oral isotretinoin. N.Eng.J.Med. 318:1633-1637, 1988.

VIII. APPENDIX A

TABLES

TABLE VIII-1: Effect of BRDU and α-MSH on proliferation rates of human melanocytes and melanoma cell lines

CELL LINE	DOUBLING TIME[a] (HOURS)		
	α-MSH[b]	CONTROL[c]	BRDU[d]
NORMAL MELANOCYTES	45 (6)	49 (8)	47 (6)
WM 35	12 (3)	11 (4)	11 (4)
WM 39	16 (5)	14 (4)	14 (3)
WM 902B	14 (3)	13 (4)	26* (5)
WM 1341D	27 (6)	26 (5)	26 (5)
SK-MEL 23	15 (4)	15 (5)	18 (3)
G 361	20 (4)	19 (6)	18 (5)

numbers in parentheses are standard deviation
a: calculated value $A_t = A_o e^{lt}$: $l = 0.693/t_2$; where t_2 is doubling time in hours.
b: cells cultivated for 48 hr. in 10^{-7} M [Nle^4,D-Phe^7]-α-MSH.
c: cells cultivated for 48 hr. in normal medium.
d: cells cultivated for 48 hr. in 1.8×10^{-5} M BRDU.
*: statistically significant difference compared to control ($p < 0.01$)

TABLE VIII-2: Effect of BRDU and α-MSH on colony forming efficiency of human melanocytes and melanoma cell lines

CELL LINE	COLONY FORMING EFFICIENCY[a]		
	α-MSH[b]	CONTROL[c]	BRDU[d]
NORMAL MELANOCYTES	0.013(0.10)	0.002(0.12)	0.008(0.10)
WM 35	0.15(0.1)	0.28(0.12)	3.47*(0.23)
WM 39	0.23(0.12)	0.19(0.13)	2.12*(0.31)
WM 902B	0.15(0.10)	0.16(0.10)	1.87*(0.24)
WM 1341D	0.37(0.23)	0.42(0.15)	0.19(0.16)
SK-MEL 23	0.15(0.13)	0.10(0.10)	2.77*(0.31)
G 361	0.23(0.15)	0.12(0.11)	1.47†(0.21)

numbers in parentheses are standard deviations
a: percent colonies per $5x10^{3}$ cells seeded.
b: cells incubated in the presence of 10^{-7} M [Nle^{4}, D-Phe^{7}]-α-MSH.
c: cells incubated in the presence of normal medium.
d: cells incubated in the presence of $1.8x10^{-5}$ M BRDU.
*: statistically significant difference compared to control ($p < 0.01$)
†: statistically significant difference compared to control ($p < 0.05$)

TABLE VIII-3: Effect of BRDU on cell migration over Collagen Type I substrate for human melanocytes and melanoma cell lines

CELL LINE	CELL MIGRATION RATE[a]	
	CONTROL[b]	BRDU[c]
NHEM	1.23 (0.02)	1.27 (0.02)
WM 35	2.79 (0.02)	5.34 (0.03)*
WM 39	1.86 (0.01)	4.68 (0.03)*
WM 1341D	3.26 (0.01)	2.68 (0.009)*
SK-MEL 23	2.59 (0.009)	5.45 (0.03)*
G 361	2.61 (0.03)	5.11 (0.04)*

numbers in parentheses are standard deviations
a: percent of total cells which migrated through membrane after 6 hr.
b: cells incubated in the presence of normal medium.
c: cells incubated in the presence of 1.8×10^{-5} M BRDU.
*: statistically significant compared to control ($p < 0.0001$)

TABLE VIII-4: Effect of BRDU and α-MSH on tyrosinase activity and melanin content of human melanocytes and melanoma cell lines.

CELL LINE	TYROSINASE ACTIVITY[a]			MELANIN CONTENT[b]		
	α-MSH[c]	CONTROL[d]	BRDU[e]	α-MSH	CONTROL	BRDU
NHEM	346 (45)	367 (54)	373 (47)	724 (65)	763 (87)	747 (65)
WM 35	124* (43)	11 (15)	26 (23)	67† (32)	30 (23)	8 (13)
WM 39	202* (57)	124 (59)	41* (21)	281* (34)	60 (21)	56 (42)
WM 902B	22 (13)	15 (13)	4 (10)	26 (13)	0 (13)	8 (16)
WM 1341D	30 (11)	22 (13)	67† (23)	82 (25)	67 (34)	674* (54)
SK-MEL 23	427 (43)	405 (65)	49* (37)	3086* (65)	2921 (59)	75* (39)
G 361	22 (11)	15 (12)	2 (12)	75 (28)	49 (25)	56 (30)

numbers in parentheses are standard deviations

a: as measured by absorbance at 405 nm (x 10^{-3})/10^{6} cells.

b: as measured by absorbance at 405 nm (x 10^{-3})/10^{6} cells.

c: cells incubated in the presence of 10^{-7} M [Nle^{4}, D-Phe^{7}]-α-MSH.

d: cells incubated in the presence of normal medium.

e: cells incubated in the presence of 1.8x10^{-5} M BRDU.

*: statistically significant difference compared to control ($p < 0.01$)

†: statistically significant difference compared to control ($p < 0.05$)

TABLE VIII-5: Corrected Median Fluorescence Intensity measuring Integrin Expression on Unexposed Human Melanocytes and Melanoma Cells

Integrin	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
α2	47.0*	113.8*	138.9*	141.6*	210.8*	107.0*	182.0*	31.6*
α3	152.9*	415.6*	287.3*	275.3*	96.0*	132.0*	202.0*	123.0*
α4	0.5	3.4	113.9*	11.7	47.9*	NT	11.5	0.7
α5	26.0	24.7	20.7	67.2*	35.6	15.0	34.0	5.8
αv	8.0	0.0	31.9	0.0	0.0	19.0	32.0	6.4
β1	303.0*	1416.8*	937.5*	1309.4*	512.6*	547.0*	352.0*	477.5*
β3	1.0	31.4	57.4	0.0	3.2	0.0	0.1	9.0
β4	18.0	1.7	0.0	0.0	0.3	2.0	2.0	0.0

corrected median fluorescence = raw median fluorescence - median fluorescence of cells using secondary antibody only

standard deviations ≤ 25 units, as calculated from 10^4 cells by FACScan II algorithm (Becton Dickenson)

Raw median fluorescence of secondary antibody only: NHEM, 37.0; WM35, 33.7; WM 39, 49.8; WM902B, 77.9; WM1341D, 51.4; SK23, 33.3; G361, 48.0; C32, 28.6

NHEM = normal melanocytes
RGP = radial growth phase
VGP = vertical growth phase
MGP = metastatic growth phase
2° Ab = secondary antibody only
* statistically significant ($p \leq 0.01$)

TABLE VIII-6: Corrected Median Fluorescence Intensity measuring Integrin Expression on Human Melanocytes and Melanoma Cells Exposed to BRDU

Integrin	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
α2	42.4*	364.9*	137.4*	279.9*	472.8*	75.13*	195.9*	139.9*
α3	146.3*	433.9*	85.2*	320.7*	938.1*	75.01*	51.2*	138.6*
α4	0.0	10.0	20.2	28.0	NT	NT	5.8	5.9
α5	22.1	142.2*	28.5	269.5	306.9*	5.8	37.0	53.2*
αv	0.5	15.0	0.5	18.1	0.0	10.0	25.8	2.9
β1	267.2*	1270.9*	197.5*	1618.2*	1798.2*	364.9*	853.9*	378.3*
β3	0.4	0.0	0.0	5.1	0.0	0.7	11.23	4.35
β4	16.5	5.5	1.3	10.5	14.8	0.2	0.3	7.2

corrected median fluorescence = raw median fluorescence - median fluorescence of cells using secondary antibody only

standard deviations ≤ 25 units, as calculated from 10^4 cells by FACScan II algorithm (Becton Dickenson)

Raw median fluorescence of secondary antibody only: NHEM, 36.5; WM35, 75.1; WM 39, 81.2; WM902B, 148.9; WM1341D, 108.4; SK23, 39.6; G361, 29.9; C32, 47.2

NHEM = normal melanocytes
RGP = radial growth phase
VGP = vertical growth phase
MGP = metastatic growth phase
2° Ab = secondary antibody only
* statistically significant ($p \leq 0.01$)

TABLE VIII-7: Corrected Median Fluorescence Intensity measuring Integrin Expression on Human Melanocytes and Melanoma Cells Exposed to α-MSH

Integrin	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
α2	52.3*	79.2*		34.1	63.4*		56.4*	58.5*
α3	163.9*	21.9		30.3	139.3		52.1*	69.9*
α4	0.4	NT		NT	NT		NT	NT
α5	27.5	12.4		25.6	33.0		24.2	28.6
αv	11.3	11.98		10.4	13.7		8.2	15.4
β1	324.0*	77.9*		83.7*	187.2*		133.2*	136.1*
β3	2.4	0.0		0.5	3.1		2.1	4.8
β4	24.0	0.0		0.0	1.5		0.0	0.8

corrected median fluorescence = raw median fluorescence - median fluorescence of cells using secondary antibody only

standard deviations ≤ 25 units, as calculated from 10^4 cells by FACScan II algorithm (Becton Dickenson)

Raw median fluorescence of secondary antibody only: NHEM, 38.5; WM35, 46.9; WM 39, WM902B, 37.9; WM1341D, 45.4; SK23, G361, 29.4; C32, 26.7

NHEM = normal melanocytes
RGP = radial growth phase
VGP = vertical growth phase
MGP = metastatic growth phase
2° Ab = secondary antibody only
* statistically significant ($p \leq 0.01$)

TABLE VIII-8: Percent Change in Integrin Expression on Normal and Malignant Melanocytes following exposure to 5-Bromodeoxyuridine as measured by Flow Cytometry

Integrin	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
2° Ab	NT	--	--	--	--	--	--	--
α2	NT	+33*	-7*	+2*	+5*	-4*	+58*	+87*
α3	NT	-49*	-43*	-30*	+236*	-16*	-48*	-26*
α4	NT	+3	-51	+3	NT	NT	-4	+9
α5	NT	+67*	+5	+51*	+126*	-10	+31	+78*
αv	NT	+26	-29	+33	+60	-8	+12	-14
ß1	NT	-58*	-75*	-33	+60*	-9*	+235*	-49*
ß3	NT	-55	-44	+8	-8	+6	+38	-17
ß4	NT	+2	+4	+60	+14	-4	-3	+25

$$\text{percent change} = \frac{\text{(median fluorescence intensity BRDU) -(median fluorescence intensity control)}}{\text{(median fluorescence intensity control)}}$$

NHEM = normal melanocytes
RGP = radial growth phase
VGP = vertical growth phase
MGP = metastatic growth phase
2° Ab = secondary antibody only
* median fluorescence intensities statistically significant (p<0.05)

Table VIII-9: Adhesion of Normal and Transformed Melanocytes Derived from Different Growth Phases of Tumour Progression to different Substrates

Substrate	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
Plastic	8.4 ± 1.0	1.3 ± 0.4	6.6 ± 2.8	3.4 ± 1.4	1.5 ± 2.1	10.8 ± 6.0	28.3 ± 2.9	2.0 ± 2.0
FN	53.7 ± 5.0	3.0 ± 0.1	70.8 ± 4.5	51.0 ± 4.9	31.7 ± 3.7	13.3 ± 1.7	38.6 ± 1.7	8.2 ± 2.0
LN	50.6 ± 3.0	4.2 ± 0.2	33.0 ± 4.5	14.0 ± 0.8	4.3 ± 1.2	20.2 ± 4.0	43.6 ± 2.7	4.8 ± 0.8
COLL I	43.9 ± 3.0	3.9 ± 0.6	53.1 ± 8.8	30.0 ± 2.7	3.9 ± 1.6	14.7 ± 4.0	48.4 ± 1.2	4.1 ± 3.0
COLL IV	48.6 ± 2.0	6.6 ± 0.7	57.2 ± 4.8	41.0 ± 3.4	6.1 ± 0.7	23.4 ± 3.5	44.0 ± 1.9	6.3 ± 0.6

NHEM = Normal melanocytes
N = Normal
RGP = Radial growth phase
VGP = Vertical growth phase
MGP = Metastatic growth phase
FN = Fibronectin
LN = Laminin
COLL = Collagen

Table VIII-10: Adhesion of Normal and Malignant Melanocytes corrected for adhesion to Plastic

Substrate	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
FN	45.3 (2.1)	1.7 (2.6)	64.2 (3.5)	47.6 (2.6)	29.7 (3.1)	2.5 (2.4)	10.3 (1.8)	9.4 (4.0)
LN	42.2 (3.1)	2.9 (2.1)	26.4 (2.4)	10.6 (1.8)	2.8 (2.8)	9.4 (2.6)	15.3 (2.9)	11.7 (2.4)
COLL I	35.5 (2.5)	2.6 (1.5)	46.5 (3.6)	26.6 (1.8)	2.4 (2.6)	3.9 (2.9)	20.1 (3.4)	13.9 (2.6)
COLL IV	40.2 (2.6)	5.3 (3.1)	50.6 (2.8)	37.6 (2.7)	4.6 (2.9)	12.6 (1.8)	15.7 (1.8)	14.8 (2.1)

Values are corrected for adhesion to blocked plastic. Determined by subtracting the following values for each cell line: NHEM: 8.4(1.0); WM35: 1.3(0.4); WM39: 6.6(2.8); WM902B: 3.4(1.4); WM1341D: 1.5(2.1); SK23: 10.8(6.0); G361: 28.3(2.9); C32: 2.0(2.0)

numbers in parentheses are standard deviations
NHEM = Normal melanocytes
N = Normal
RGP = Radial growth phase
VGP = Vertical growth phase
MGP = Metastatic growth phase
FN = Fibronectin
LN = Laminin
COLL = Collagen

Table VIII-11: **Percent Difference in Adhesion Strength between Normal and Malignant Melanocytes in the Presence and Absence of BRDU**

Substrate	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
Plastic	NT	+8.8	+3.9	-3.4	+8.4	-8.6	-28.3	-2.0
FN	NT	+24.3	-9.7	-19.6	+41.4	+1.3	-31.6	+9.4
LN	NT	+14.6	+4.5	-2.6	+22.2	+10.7	+7.8	+11.7
COLL I	NT	+22.0	-4.3	-9.5	+26.4	+34.2	-42.5	+13.9
COLL IV	NT	+22.2	+5.3	-17.4	+45.7	+43.2	-26.3	+14.8

$$\text{percent change} = \frac{\text{(percent adhesion BRDU) -(percent adhesion control)}}{\text{(percent adhesion control)}}$$

NHEM = normal melanocytes
RGP = radial growth phase
VGP = vertical growth phase
MGP = metastatic growth phase

Table VIII-12: Effect of Blocking Antibodies on Cell-Substrate Adhesion (Percent Adhesion)

Integrin	Fibronectin		Laminin		Collagen Type I		Collagen Type IV	
	NHEM	**G 361**	**NHEM**	**G 361**	**NHEM**	**G 361**	**NHEM**	**G 361**
Control	52.6 5	40.2 1.5	47.3 3.0	47.2 3.4	45.6 3.4	47.6 2.8	48.6 2.0	45.0 2.3
α2	48.9 3.2	41.0 3.7	28.4 5.6	21.2 6.2	31.0 2.3	30.5 3.2	24.3 1.5	26.1 2.5
α3	49.4 6.7	34.2 4.2	16.1 2.3	13.2 5.2	43.8 2.3	45.2 3.5	47.1 2.5	43.2 3.5
α4	51.0 5.1	41.8 1.8	44.9 4.7	45.3 8.3	45.1 3.6	46.2 2.7	44.2 2.6	42.3 3.5
α5	16.3 3.3	10.5 1.7	44.5 8.3	41.1 4.8	42.0 1.2	47.1 1.8	45.2 2.4	44.6 2.8
αv	48.9 4.2	38.6 3.2	44.6 6.5	43.9 4.6	42.4 3.5	46.2 3.6	46.7 3.4	41.9 2.9
ß1	21.0 2.8	17.3 2.6	2.8 1.7	3.8 2.1	1.8 1.0	2.4 1.2	2.9 1.3	4.5 2.0
ß3	50.0 3.8	38.6 3.5	44.0 3.7	41.1 6.2	41.9 2.6	47.1 3.9	47.2 1.6	44.1 2.8
ß4	44.7 4.7	39.4 2.9	10.9 2.6	41.1 5.6	42.4 2.8	44.7 2.1	47.2 3.5	42.3 3.5

upper number is percent adhesion, lower number is standard deviation

NHEM = Normal human epidermal melanocytes

Table VIII-13: Effect of Anti-Integrin Antibodies on Cell-Substrate Adhesion by Normal and Malignant Melanocytes (Percent of Control Adhesion)

Integrin	Fibronectin		Laminin		Collagen Type I		Collagen Type IV	
	NHEM	**G 361**	**NHEM**	**G 361**	**NHEM**	**G 361**	**NHEM**	**G 361**
Control	100	100	100	100	100	100	100	100
α2	93 6	102 9	60 12	45 12	68 5	64 7	56 5	58 8
α3	94 12	85 10	35 5	28 11	96 8	95 7	97 6	96 5
α4	97 9	104 5	95 10	96 14	99 10	97 10	91 6	94 12
α5	31 6	26 5	94 15	87 10	96 10	99 12	93 12	99 6
αv	93 8	96 8	94 12	93 10	93 12	97 12	96 11	93 8
ß1	40 5	43 6	6 4	8 5	4 5	5 6	6 6	10 9
ß3	95 7	96 9	93 8	87 13	92 12	99 6	97 8	98 9
ß4	85 9	98 7	23 5	87 12	93 11	94 12	97 10	94 10

upper number is percent of cell adhesion compared to control, lower number is standard deviation

NHEM = Normal human epidermal melanocytes

Table VIII-14: Effect of Divalent Cations on Adhesion to Fibronectin by Normal and Malignant Melanocytes (Percent Adhesion)

Cation	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
EDTA	2.0 0.2	6.7 1.1	16.9 2.7	33.7 1.0	1.7 1.1	5.0 4.0	3.8 1.0	13.7 7.0
Calcium	9.2 1.0	4.5 0.7	65.7 4.2	44.0 0.6	4.7 1.5	1.1 1.0	8.1 2.0	21.2 5.0
Magnesium	23.5 2.5	5.4 0.8	66.7 3.1	47.4 3.0	16.2 3.0	9.1 3.0	35.1 2.5	20.9 1.8
Manganese	58.1 1.5	84.9 4.3	98.0 2.3	57.9 9.9	73.9 5.9	17.8 3.0	55.1 1.9	47.5 2.0
Mn/Mg	27.9 2.5	74.5 4.1	100.0 2.0	91.2 8.9	89.1 5.2	36.6 3.0	52.9 1.5	39.5 2.0
Mg/Ca	53.7 2.5	3.0 0.1	70.8 4.5	51.0 4.9	31.7 3.7	13.3 1.7	38.6 1.7	8.2 2.0

upper number is percent adhesion, lower number is standard deviation

N = Normal
NHEM = Normal human epidermal melanocytes
RGP = Radial growth phase
VGP = Vertical growth phase
Mn = Manganese
Mg = Magnesium
Ca = Calcium

Table VIII-15: Effect of Divalent Cations on Adhesion to Laminin by Normal and Malignant Melanocytes (Percent Adhesion)

Cation	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
EDTA	3.5 0.2	5.7 0.5	7.7 3.4	13.5 1.9	0.3 0.9	1.3 1.0	6.9 2.0	7.1 3.0
Calcium	5.7 1.0	4.5 0.7	15.4 5.0	16.8 3.0	1.5 0.3	7.1 3.0	38.7 1.5	15.0 5.0
Magnesium	25.2 2.0	17.4 5.8	21.0 1.6	15.3 2.3	1.5 0.6	22.2 3.0	39.6 2.7	10.6 1.3
Manganese	67.6 1.5	40.4 5.0	61.1 4.0	39.3 5.7	17.5 3.2	25.9 2.0	54.7 0.6	32.0 1.7
Mn/Mg	48.1 2.5	41.9 3.8	79.4 11.4	78.5 7.8	21.6 3.2	30.6 2.8	53.5 1.0	18.2 3.3
Mg/Ca	50.6 3.0	4.2 0.2	33.0 4.5	14.0 0.8	4.3 1.2	20.2 4.0	43.6 2.7	4.8 0.8

upper number is percent adhesion, lower number is standard deviation

N = Normal
NHEM = Normal human epidermal melanocytes
RGP = Radial growth phase
VGP = Vertical growth phase
Mn = Manganese
Mg = Magnesium
Ca = Calcium

Table VIII-16: Effect of Divalent Cations on Adhesion to Collagen Type I by Normal and Malignant Melanocytes (Percent Adhesion)

Cation	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
EDTA	2.0 0.3	4.4 0.2	14.3 3.7	21.3 1.0	0.0 0.4	0.7 1.0	3.9 1.0	7.7 3.0
Calcium	3.0 1.2	4.1 1.3	13.5 3.1	28.3 2.2	2.2 1.3	3.8 2.0	3.2 2.0	18.8 7.0
Magnesium	30.2 2.0	5.5 0.4	45.4 2.6	36.9 6.9	0.0 0.7	13.1 2.0	43.7 0.9	12.4 2.0
Manganese	59.4 2.0	32.9 3.1	80.9 4.4	42.2 6.3	22.2 2.0	18.2 4.0	56.2 1.3	30.4 4.0
Mn/Mg	37.5 3.0	30.9 2.8	90.4 8.9	81.3 6.5	21.3 8.2	32.9 5.0	50.4 1.7	24.1 0.4
Mg/Ca	43.9 3.0	3.9 0.6	53.1 8.8	30.0 2.7	3.9 1.6	14.7 4.0	48.4 1.2	4.1 3.0

upper number is percent adhesion, lower number is standard deviation

N = Normal
NHEM = Normal human Epidermal melanocytes
RGP = Radial growth phase
VGP = Vertical growth phase
Mn = Manganese
Mg = Magnesium
Ca = Calcium

Table VIII-17: Effect of Divalent Cations on Adhesion to Collagen Type IV by Normal and Malignant Melanocytes (Percent Adhesion)

Cation	N	RGP	VGP			Metastatic		
	NHEM	WM35	WM39	WM902B	WM1341D	SK23	G361	C32
EDTA	1.5 0.3	3.7 0.5	10.2 1.4	29.2 3.3	0.0 0.8	1.3 1.0	2.5 1.0	6.0 4.0
Calcium	5.4 0.6	4.0 0.4	12.1 2.8	25.3 3.0	2.9 0.5	6.5 3.0	5.5 1.3	7.2 1.6
Magnesium	33.6 3.0	8.6 0.8	43.4 3.6	34.9 2.8	1.3 0.5	22.3 1.5	38.0 2.4	12.3 2.5
Manganese	79.2 3.0	22.3 2.5	68.4 3.5	30.5 1.5	24.4 2.2	22.0 3.0	54.7 2.1	21.7 3.0
Mn/Mg	44.7 2.5	24.3 3.9	87.4 12.2	88.7 9.7	17.5 4.2	32.8 4.0	51.0 2.0	21.2 4.0
Mg/Ca	48.6 2.0	6.6 0.7	57.2 4.8	41.0 3.4	6.1 0.8	23.4 3.5	44.0 1.9	6.3 0.6

upper number is percent adhesion, lower number is standard deviation

N = Normal
NHEM = Normal human epidermal melanocytes
RGP = Radial growth phase
VGP = Vertical growth phase
Mn = Manganese
Mg = Magnesium
Ca = Calcium

Table VIII-18: Effect of Manganese on Percent Change in Cell-Substrate Adhesion by Normal and Malignant Melanocytes

Substrate	N	RGP	VGP			MGP	
	NHEM	WM35	WM1341D	WM902B	WM39	SK23	G361
FN	+9	+2730*	+38	+13	+133*	+34	+42
LN	+33	+862*	+307*	+180*	+85*	+28	+25
COLL I	+35	+744*	+469*	+40	+52	+24	+16
COLL IV	+63*	+238*	+300*	-25	+19	-5	+24

$$\text{percent change} = \frac{\text{(percent adhesion BRDU) -(percent adhesion control)}}{\text{(percent adhesion control)}}$$

N = Normal
NHEM = normal melanocytes
RGP = radial growth phase
VGP = vertical growth phase
MGP = metastatic growth phase
FN = Fibronectin
LN = Laminin
COLL = Collagen
* $p \leq 0.05$

Table VIII-19: Effect of BRDU on Percent Change in Cell-Substrate Adhesion by Normal and Malignant Melanocytes following Manganese Treatment

Substrate	N	RGP	VGP			MGP	
	NHEM	WM35	WM1341D	WM902B	WM39	SK23	G361
FN	NT	+32	+3	-34	0	+55	+1050*
LN	NT	+18	+34	-13	+38	+16	-6
COLL I	NT	0	-24	-47	+18	-12	+45
COLL IV	NT	-5	-26	+33	-21	-38	+133

$$\text{percent change} = \frac{\text{(percent adhesion BRDU) -(percent adhesion control)}}{\text{(percent adhesion control)}}$$

N = Normal
NHEM = normal melanocytes
RGP = radial growth phase
VGP = vertical growth phase
MGP = metastatic growth phase
FN = Fibronectin
LN = Laminin
COLL = Collagen
* $p \leq 0.05$

TABLE VIII-20: Effect of Divalent Cations on Cell-Substrate Adhesion by Normal Melanocytes: Control

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
PLA	2.50	4.00	9.40	25.00	7.10	8.40
	0.3	0.8	1.3	0.5	1.5	1.0
FN	2.00	9.20	23.50	58.10	27.90	53.70
	0.2	1.0	2.5	1.5	2.5	2.5
LN	3.50	5.70	25.20	67.60	48.10	50.60
	0.2	1.0	2.0	1.5	2.5	3.0
COLL I	2.00	3.00	30.20	59.40	37.50	43.90
	0.3	1.2	2.0	2.0	3.0	3.0
COLL IV	1.50	5.40	33.60	79.20	44.70	48.60
	0.3	0.6	3.0	3.0	2.5	2.0

TABLE VIII-21: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 35 Melanoma: Control

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	5.10	4.30	2.30	14.90	26.60	1.30
	0.8	0.8	0.6	3.6	4.6	0.4
FN	6.70	4.50	5.40	84.90	74.50	3.00
	1.1	0.7	0.8	4.3	4.1	0.1
LN	5.70	4.50	17.40	40.40	41.90	4.20
	0.6	0.7	5.8	5.0	3.8	0.2
COLL I	4.40	4.10	5.50	32.90	30.90	3.90
	0.2	1.3	0.4	3.1	2.8	0.6
COLL IV	3.70	4.00	8.60	22.30	24.30	6.60
	0.5	0.4	0.8	2.5	3.9	0.7

TABLE VIII-22: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 35 Melanoma: BRDU

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	8.90	23.50	9.40	19.20	17.20	10.10
	0.5	5.0	1.5	1.5	2.0	3.0
FN	11.40	42.50	17.00	36.00	42.10	27.30
	1.5	2.5	2.0	5.0	4.0	4.0
LN	13.40	41.10	12.50	22.10	36.20	18.80
	1.5	2.5	2.0	1.0	2.0	2.5
COLL I	16.30	15.20	12.90	25.50	30.90	25.90
	0.7	5.0	3.0	3.5	3.5	3.5
COLL IV	17.0	13.60	15.90	27.30	28.80	28.80
	3.0	3.0	1.5	1.5	4.5	5.0

TABLE VIII-23: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 35 Melanoma: MSH

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	5.10	10.40	10.90	10.80	48.10	7.10
	0.4	1.5	0.8	2.0	4.0	1.5
FN	4.70	14.60	10.00	24.30	31.40	9.20
	2.0	1.5	1.5	1.5	3.5	2.0
LN	5.10	22.50	11.7	16.3	20.90	14.20
	1.0	2.0	2.0	2.5	3.0	2.5
COLL I	4.00	17.60	8.40	15.90	21.30	8.40
	1.0	2.0	1.5	2.5	2.0	1.5
COLL IV	1.10	15.50	8.40	11.30	16.30	3.30
	1.5	1.0	1.0	2.0	1.5	1.0

TABLE VIII-24: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 39 Melanoma: Control

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	3.50	1.20	3.20	40.90	71.80	6.60
	1.2	0.8	0.8	9.1	4.7	2.8
FN	16.90	65.70	66.70	98.0	100.00	70.80
	2.7	4.2	3.1	2.3	2.0	4.5
LN	7.70	15.40	21.00	61.10	79.40	33.00
	3.4	5.0	1.6	4.0	11.4	4.5
COLL I	14.30	13.50	45.40	80.90	90.40	53.10
	3.7	3.1	2.6	4.4	8.9	8.8
COLL IV	10.20	12.10	43.40	68.40	87.40	57.20
	1.4	2.8	3.6	3.5	12.2	4.8

TABLE VIII-25: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 39 Melanoma: BRDU

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	0.00	0.00	0.10	34.30	43.40	10.50
	0.5	0.5	0.1	6.9	5.4	5.0
FN	19.90	44.10	50.00	61.00	80.50	61.10
	0.7	0.7	2.1	6.7	10.0	5.0
LN	8.80	18.60	30.40	51.90	73.80	37.50
	0.8	1.3	0.9	6.7	4.2	4.0
COLL I	16.60	26.50	38.50	57.30	76.70	48.80
	0.7	5.6	3.3	3.8	4.1	1.3
COLL IV	22.20	28.10	41.50	49.30	73.20	62.50
	1.0	3.3	4.1	2.0	5.9	7.3

TABLE VIII-26: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 39 Melanoma: MSH

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	5.1	4.1	4.0	21.0	11.8	19.2
	0.1	1.7	0.9	8.0	2.6	2.7
FN	5.5	46.8	50.8	86.1	69.9	40.1
	0.9	7.4	5.8	8.0	6.6	6.4
LN	4.5	6.5	2.9	10.3	12.1	5.9
	0.3	1.4	1.2	1.4	1.6	0.6
COLL I	6.3	6.7	2.9	10.3	12.1	5.9
	2.0	0.5	1.2	1.4	1.6	0.6
COLL IV	4.1	7.0	17.2	29.0	28.8	17.6
	0.7	1.4	3.9	2.7	1.3	5.2

TABLE VIII-27: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 902B Melanoma: Control

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	10.7	6.2	5.7	19.6	53.8	3.4
	3.4	3.1	2.3	10.0	12.6	1.4
FN	33.7	44.0	47.4	57.9	91.2	51.0
	1.0	0.6	3.0	9.9	8.9	4.9
LN	13.5	16.8	15.3	39.3	78.5	14.0
	1.9	3.0	2.3	5.7	7.8	0.8
COLL I	21.3	28.3	36.9	42.2	81.3	30.0
	1.0	2.2	6.9	6.3	6.5	2.7
COLL IV	29.2	25.3	34.9	30.5	88.7	41.0
	3.3	3.0	2.8	1.5	9.7	3.4

TABLE VIII-28: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 902B Melanoma: BRDU

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	5.9	0.6	0.0	0.0	28.3	0.0
	1.0	0.4	0.5	0.5	1.6	0.5
FN	33.0	30.5	27.8	20.6	58.4	31.4
	3.7	2.2	4.4	3.9	3.1	0.8
LN	23.3	14.2	13.3	9.9	41.9	11.4
	8.0	2.8	0.5	3.1	2.5	3.4
COLL I	22.2	21.6	17.0	10.7	43.2	20.5
	1.8	3.5	3.2	4.4	4.6	1.5
COLL IV	35.9	28.9	25.2	31.4	47.2	23.6
	9.0	10.0	6.8	10.0	7.3	4.1

TABLE VIII-29: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 1341D Melanoma: Control

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	2.2	2.5	2.0	9.1	8.5	1.5
	3.3	1.9	0.5	3.0	3.4	2.1
FN	1.7	4.7	16.2	73.9	89.1	31.7
	1.1	1.5	3.0	5.9	5.2	3.7
LN	0.3	1.5	1.5	17.5	21.6	4.3
	0.9	0.3	0.6	3.2	3.2	1.2
COLL I	0.0	2.2	0.0	22.2	21.3	3.9
	0.4	1.3	0.7	2.0	8.2	1.6
COLL IV	0.0	2.9	1.3	24.4	17.5	6.1
	0.8	0.5	0.5	2.2	4.2	0.75

TABLE VIII-30: Effect of Divalent Cations on Cell-Substrate Adhesion by WM 1341D Melanoma: BRDU

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	2.2	1.6	2.4	19.9	35.8	9.9
	0.4	0.9	1.6	9.8	5.2	5.5
FN	4.9	24.6	66.3	75.5	98.2	73.1
	1.0	7.9	16.2	7.1	5.1	12.3
LN	4.5	11.5	13.0	35.5	42.8	26.5
	0.4	6.8	2.9	6.6	6.5	10.6
COLL I	3.0	2.2	8.5	22.9	31.8	30.3
	0.8	0.9	3.7	0.5	1.4	1.5
COLL IV	3.4	4.2	9.8	38.3	31.0	51.8
	0.8	1.1	1.6	4.1	8.1	11.6

TABLE VIII-31: Effect of Divalent Cations on Cell-Substrate Adhesion by G 361 Melanoma: Control

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	3.6	4.0	5.7	55.5	52.2	28.3
	1.0	0.5	0.8	0.6	1.2	2.9
FN	3.8	8.1	35.1	55.1	52.9	38.6
	1.0	2.0	2.5	1.9	1.5	1.7
LN	6.9	38.7	39.6	54.7	53.5	43.6
	2.0	1.5	2.7	0.6	1.0	2.7
COLL I	3.9	3.2	43.7	56.2	50.4	48.4
	1.0	2.0	0.9	1.3	1.7	1.2
COLL IV	2.5	5.5	38.0	54.7	51.0	44.0
	1.0	1.3	2.4	2.1	2.0	1.9

TABLE VIII-32: Effect of Divalent Cations on Cell-Substrate Adhesion by G 361 Melanoma: BRDU

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	1.3	0.7	10.3	15.2	39.2	0.0
	0.5	1.1	2.8	2.9	9.9	0.5
FN	1.7	0.4	9.4	80.7	94.0	7.0
	1.1	0.5	2.7	8.2	9.8	3.1
LN	2.7	9.2	29.2	48.3	81.5	51.4
	0.8	1.6	4.3	4.9	9.5	5.8
COLL I	0.0	0.2	7.6	32.1	64.1	5.9
	1.6	0.5	2.7	9.6	5.9	4.5
COLL IV	0.6	0.8	5.7	41.3	48.3	17.7
	0.7	1.2	1.0	7.8	10.6	5.4

TABLE VIII-33: Effect of Divalent Cations on Cell-Substrate Adhesion by SK-MEL 23 Melanoma: Control

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	3.6	1.1	0.7	17.4	18.5	10.8
	2.0	1.0	1.0	3.0	3.0	6.0
FN	5.0	1.1	9.1	17.8	36.6	13.3
	4.0	1.0	3.0	3.0	3.0	1.7
LN	1.3	7.1	22.2	25.9	30.6	20.2
	1.0	3.0	3.0	2.0	2.8	4.0
COLL I	0.7	3.8	13.1	18.2	32.9	14.7
	1.0	2.0	2.0	4.0	5.0	4.0
COLL IV	1.3	6.5	22.3	22.0	32.8	23.4
	1.0	3.0	1.5	3.0	4.0	3.5

TABLE VIII-34: Effect of Divalent Cations on Cell-Substrate Adhesion by SK-MEL 23 Melanoma: BRDU

Substrate	EDTA	Ca	Mg	Mn	Mn/Mg	Mg/Ca
Plastic	1.7	0.0	2.8	11.5	12.8	2.2
	0.8	0.3	1.1	1.1	2.6	0.1
FN	0.0	0.0	4.3	22.7	46.4	14.6
	0.3	0.3	0.8	3.0	2.2	2.0
LN	2.2	0.8	12.9	36.0	45.9	30.9
	0.8	0.5	1.1	2.8	4.5	4.0
COLL I	0.0	0.0	26.1	42.7	50.6	48.9
	1.4	0.3	4.0	0.6	2.4	1.2
COLL IV	0.0	0.0	36.0	41.1	49.3	66.6
	0.6	0.4	1.0	2.0	5.0	6.1

IX. APPENDIX B

DIVALENT CATIONS CONTROL CELL-SUBSTRATE ADHESION IN NORMAL AND MALIGNANT HUMAN MELANOCYTES[*]

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ABSTRACT

Integrins are an adhesion molecule class that depends on divalent cations for proper function. This study examined whether normal melanocytes and malignant melanocytes expressing early and late differentiation markers would differ in adhesion to substrates, and whether divalent cations could influence this adhesive ability. Adhesion of normal human melanocytes, and metastatic malignant melanoma cell lines expressing early (G361) and late (SK-MEL23) differentiation markers was determined on fibronectin, laminin, collagens type I and type IV using a quantitative adhesion assay. Integrin subunit expression was determined by flow cytometry. Integrin-specific adhesion was determined using soluble GRGDS peptide and subunit-specific functional blocking antibodies. This study shows that both normal and malignant melanocytes adhere to extracellular matrices in a divalent cation-dependent manner, and adhesion strength varies with cation species. Integrins can be rapidly activated by small alterations in cation concentration, with manganese-containing solutions being the most potent. There were marked differences in substrate adhesion between normal melanocytes and metastatic malignant melanoma cells. The three cell types, however, expressed the same integrin subunits in approximately equal proportions. Thus, both normal and malignant human melanocytes require divalent cations for adhesion to extracellular matrices, and the degree of substrate adhesion is different between them and sensitive to the cation species present. Manganese, in particular, appears to cause adhesion by activating both integrin-dependent and independent mechanisms.

INTRODUCTION

Cell adhesion to extracellular matrix molecules uses a family of cell surface molecules called integrins, which are α-ß heterodimers that integrate the extracellular matrix with the cell cytoskeleton via the ß-subunit [Ruoslahti and Pierschbacher, 1987]. The α-subunit appears to direct ligand specificity by using divalent cation-binding domains similar to the "EF-hand" structural motif found in the divalent cation binding regions on the enzyme calmodulin [Edwards et al, 1988]. These domains cause conformational changes in the molecule, which may activate the receptor by uncovering active epitopes [Grzesiak et al, 1992]. It is not known how cells utilize these conformational changes to selectively bind to substrates, or whether this ability can vary with the state of cell differentiation.

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Human malignant melanoma may provide a model for studying cell differentiation and adhesion *in vivo* and *in vitro*, and the latter can be best illustrated by comparing with non-metastatic normal melanocytes [see Clark et al, 1984 for review]. Normal non-metastatic and malignant metastatic melanocytes differ in their mobility and adhesion to extracellular matrix, presumably due to differences in adhesion molecule expression and integration with the cytoskeleton. However, it is not known to what extent integrin expression changes according to the cellular differentiation state, including metastatic potentials, and how such alterations in integrin expression affect adhesion to substrates.

This study compares integrin expression and adhesion to extracellular matrices between normal and malignant human melanocytes. Specifically, we were interested in determining whether divalent cations could influence adhesive ability to extracellular matrix substrates. Towards this end, we developed a new modification of a quantitative, colourimetric adhesion assay, which is different from the methods of McClay [McClay et al, 1981] and Lotz and her colleagues [1989]. Secondly, by using this assay, we have determined whether the state of cell differentiation and/or malignant transformation (e.g., metastatic versus non-metastatic) affects integrin expression, or substrate adhesion in the presence of divalent cations. In addition to normal human melanocytes, a nonadherent small cell lung carcinoma cell line, and two human melanoma cell lines representing early or late differentiation state were examined. The two melanoma cell lines derive from highly metastatic clones, but differ in their capacity for melanin synthesis; one (G361 cell line) being amelanotic (early differentiation stage) and another (SK-MEL23 cell line) being highly pigmented (late differentiation stage). This study shows that, while all cells can adhere to extracellular matrices, the cation species can greatly alter this adhesion ability in a cell-specific manner which is independent of the stage of cellular differentiation. Furthermore, it appears that manganese constitutively activates integrins in the absence of ligand, and initiates a "post-integrin" cell adhesion cascade through non-integrin mechanisms. Our findings support a model of cell-cell adhesion in which an integrated cascade of multiple, cooperative adhesion systems occur, each informing the cells about their microenvironment, and directing their behaviour (e.g., attachment and spreading).

MATERIALS AND METHODS

Materials

Fibronectin, laminin, collagen type IV, ethylenediamine tetraacetic acid (EDTA), trypsin/EDTA, trichloroacetic acid, and MTT were obtained from Sigma (St. Louis, MO). Collagen type I was obtained from Upstate Biologicals Inc. (Rochester, NY). Nunc Polysorp F-16 wells, and Dulbecco's modified essential medium (DMEM) were purchased from Gibco (Montreal, PQ). Nitrocellulose membrane, and the silicone gasket and pressure plate fashioned from a large vertical gel electrophoresis apparatus were obtained from Bio-Rad (Mississauga, ON). MCDB 153 media was purchased from Clonetics (San Diego, CA). Dimethyl sulfoxide (spectrophotometric grade) was obtained from Aldrich (Milwaukee, WI). The nonfat skim milk powder came from Carnation (Leamington, ON).

The small cell lung carcinoma cell line, H-69, was obtained from the American Type Culture Collection (ATCC: Rockville, MD). Two human melanoma cell lines were used in this study, G361 was obtained from ATCC, while SK-MEL23 was a generous gift of A. Houghton (Memorial Sloan Kettering Cancer Institute, New York, NY).

Monoclonal antibodies against the human α2-integrin [P1E6], α3-integrin [P1B5], α4-integrin [P4G9], α5-integrin [P1D6], αv-integrin [VNR 147], ß2-integrin [P4H9], ß4-integrin [3E1] were generously supplied from Telios (Seattle, WA). Monoclonal antibodies against the ß1-integrin [4B4] subunit was obtained from Coulter (Mississauga, ON). A secondary goat anti-murine antibody conjugated with phycoerythrin was obtained from Becton-Dickenson (Mississauga, ON) and was used for flow cytometry studies (see below).

In vitro culture of human melanocytes and melanoma cells.

Human epidermal melanocytes were isolated according to the method of Eisinger and Marko [1982]. All cell lines were grown in 75 cm^2 tissue culture flasks (Corning, Mississauga, ON). They were grown in F-10 medium supplemented with 5% (v/v) fetal bovine serum (FBS) (Hyclone, Seattle, WA), bovine pituitary extract (2 µl/ml), phorbol 12,13-dibutyrate (2 µg/ml), basic fibroblast growth factor (2 µg/ml), and penicillin/streptomycin (10 µl/ml) (100x solution, Gibco, Montreal,PQ). Fibroblast overgrowth was eliminated by treating the cultured cells with Geneticin (100 µg/ml) (Sigma, St. Louis, MO) [Halaban and Alfano, 1984].

Human melanoma cell lines of G361 and SK-MEL23 and the small cell lung carcinoma cell line, H-69, were grown in DMEM supplemented with 5% FBS and penicillin/streptomycin (10 μg/ml) at 5% CO_2 at 37°C.

Cell passage was performed using 0.1 M phosphate buffered saline (PBS) (pH 7.4) containing 10 mM EDTA (EDTA/PBS), in order to protect the cell surface molecules from proteolysis.

Flow cytometry analysis of integrin expression on melanocytes

Human melanocytes and melanoma cells detached from the culture flask with EDTA/PBS were pelleted and resuspended in 0.1M PBS at 4°C. All steps were performed at 4°C. Aliquots of cell suspensions were placed into 1.5 ml Eppendorf centrifuge tubes (Rose Scientific, Edmonton, AB) and gently pelleted at 200 g for 30 sec. Cells were resuspended in 0.1M PBS containing monoclonal antibodies against integrin subunits at 1/50 dilution for 30 min. The cells were pelleted, the supernatant was discarded by aspiration, and the pellet was resuspended in a phycoerythrin-conjugated secondary antibody diluted 1/20 in 0.1M PBS for 30 min. The cells were pelleted, the supernatant was discarded by aspiration, and the pellet was washed 3 times in 0.1M PBS, and then fixed with 1% paraformaldehyde in 0.1M PBS. Log fluorescence intensity was measured on a FACScan flow cytometer (Becton-Dickenson, Mississauga, ON). Statistical comparison between experimental and control median fluorescence was determined by Student t-distribution.

Immunohistochemistry of extracellular matrix expression in vitro

Cells were cultured on glass coverslips in the presence of divalent cations in Tris-saline buffer (pH 7.3) for one hr. Cells were fixed with 4% paraformaldehyde in PBS (Ph 7.4) for 10 min. After washing 5 times in PBS, the cells were blocked with 0.7% lambda carageenan in 0.1% Triton X-100/PBS (pH 7.4) for 30 min [Sofroniew and Schrell, 1982]. Primary antibodies against fibronectin, laminin, or collagen type IV (1:100) were incubated for 30 min. After extensive washing in PBS, the horseradish peroxidase-conjugated goat anti-mouse secondary antibody was incubated for 30 min. After washing, the colourimetric reaction was performed using diaminobenzadine (1 mg/ml) and 0.1% hydrogen peroxide for 10 min. An image analyzer (CAS 200, Cell Analysis Systems, Inc, Elmhurst, IL) using digital analysis software (Cell Measurement Program, ver. 1.0, Cell Analysis Systems, Inc, Elmhurst, IL) was used to calculate the Sum of Optical Densities, and the Average Optical Density for 100 cells randomly selected. Statistical comparison between means was determined by Student t-distribution. Alternatively, a fluorescein-conjugated anti-mouse secondary antibody was incubated for 30 min. Cells were photographed using a Ziess Photomicroscope III using Tri-X 400 film (Kodak, Rochester, NY).

Coating of microwells

The microwells were coated with one of the following substrates dissolved in 0.1 M PBS (Ph 7.4): poly-L-lysine (0.01%), fibronectin (10 μg/ml), laminin (10 μg/ml), and collagen Type IV (10 μg/ml). The wells were loaded with 50 μl of each substrate solution and incubated at 37 °C. for 60 min. After rinsing twice with PBS/10 Mm EDTA, all nonspecific adhesive sites were blocked with 10% nonfat milk powder (Carnation, Leamington, ON) incubated for 60 min at 37 °C. Milk was selected as the blocking agent of choice after testing several other agents (see below). The wells were rinsed twice again with PBS/10 mM EDTA before the addition of pigment cells.

Enzyme-linked immunosorbance assay (ELISA) of substrate adsorption to plastic

Microwells were coated with 10% (w/v) nonfat milk powder in PBS (pH 7.4) for 30 min at 37°C. After rinsing with PBS, the wells were rinsed for 10 min in 10 mM EDTA/PBS, and then rinsed again with PBS to remove at traces of EDTA. Extracellular matrix substrates at the concentrations described above were incubated on the blocked plastic for 60 min at 37°C., then the microwells were rinsed with PBS x 3. Primary antibodies against fibronectin, laminin, or collagen type IV (1:100) were incubated for 30 min. After extensive washing in PBS, the horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:100) was incubated for 30 min. After washing, the colourimetric reaction was performed using ABTS (20 μg/ml) and 0.1% hydrogen peroxide for 10 min. The absorbance was read on a multiwell plate reader (SLT Industries, Salzberg, Austria) at 405 nm. Mean values and standard deviation from six replicates were calculated. Statistical comparison between means was determined by Student t-distribution.

Sulforhodamine B assay

A cell quantification method using a colourimetric measurement of cellular protein was used to determine cell-substrate adhesion. The sulforhodamine B (SRB) assay was performed according to the method of Skehan and colleagues [1990]. A stock solution was prepared at a concentration of 0.4% (w/v) in 1% acetic acid, and filtered through a 0.22 μm filter (Millipore, Mississauga, ON) to remove any particulate matter.

Briefly, adherent cells in microwells following plate washing was precipitated using ice cold trichloroacetic acid (10%) for one hour at 4°C. The plates were washed with running tap water and dried in a 65°C. oven for 10 minutes. Fifty µl of stock SRB solution was added to each microwell and incubated for 30 min at room temperature. The plates were washed 4 times with 1% acetic acid and flicked dry. The plates were completely dried in the oven for 10 min. Colourimetric development was performed using 50 µl of 10 mM Tris (pH 10.5), and incubated on an orbital shaker for 30 min at room temperature. Absorbance at 550 nm was measured on an ELISA microwell plate reader (SLR Industries, Salzberg, Austria). The net absorbance was determined by subtracting the absorbance of blank cell-free wells filled with 50 µl of 10 mM Tris buffer.

Quantitative Cell Adhesion Assay

The method was a modification of the method reported by Lotz and colleagues [1989]. The pigment cells were detached with 10mM EDTA/PBS. The cells were pelleted by centrifugation at 200 g at room temperature in a Beckman TJ-6 centrifuge (Beckman, Mississauga, ON), and resuspended in serum-free media. The cells were plated on precoated microwells at $5x10^4$/100 µl. The cells were then briefly centrifuged (200g) at room temperature for 2 min in the TJ-6 centrifuge with a swinging bucket rotor in order to bring cells into contact with the plate surface. The cells were incubated for the indicated time periods in an incubator (37 °C, 5% CO_2). The wells were gently filled to the top with fresh media prewarmed to 37°C, and a nitrocellulose membrane strip premoistened with media was placed over the wells, taking care to exclude all air bubbles. The silicone rubber gasket and pressure plate were placed over the nitrocellulose, and clamped using a bulldog stationery clamp. The gasket was fashioned from the silicone rubber strip that sealed the bottom of the gel plates in the casting stand. The pressure plate was made from a single thick gel plate spacer used to make large format gels. Both the gasket and the spacer were cut into two lengths of 10 cm each. The apparatus was then placed in the TJ-6 centrifuge in an inverted position with the nitrocellulose on the bottom of the bucket, and centrifuged for 8 min at three different speeds; 500 rpm (54xg), 1000 rpm (212xg), or 2000 rpm (854xg). The apparatus was disassembled and the strip carefully peeled from the wells, placed over a new 96-well plate and the impressions caused by the wells of the original plate containing the detached cells were aligned with the microwells and punched into the wells using a flame-burnished glass rod with an outside diameter equal to the inside diameter of the microwell. The SRB assay was performed on the cells contained in the two microwell plates (see above). Results were expressed as the mean and standard deviation of six-duplicate determinations.

Perturbation studies on cell adhesion

Divalent cations were selectively added or deleted from the incubation media to determine the effect of these cations on cell adhesion. Adhesion strength was measured on three substrates: laminin (10 μg/ml), fibronectin (10 μg/ml), collagen type I (10μg/ml), and collagen type IV (10 μg/ml). Standard assay conditions were used as described above, except the cells were incubated on the substrate for 1 hr in 0.1M PBS (pH 7.4), containing one of the following: 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, or 1 mM $MgCl_2$ and 1 mM $MnCl_2$.

Selective blockade of integrin subunits was achieved by adding to the incubation medium monoclonal antibodies against specific integrin subunits which are known to block functional activity. Cells were examined using the standard assay conditions as described above, except that cells were pretreated in serum-free media containing a 1/20 dilution of antibody , followed by a 1 hr incubation in the precoated microwells in serum-free media containing 1/100 dilution of antibody.

RESULTS

Development of modified centrifugation detachment assay

The inverted centrifugation detachment assay of McClay provided a reproducible and rapid method of measuring cell detachment by applying a vertical detachment force. Our method improved the quantitation of cell numbers by using the following modifications: 1) detached cells were captured on a nitrocellulose membrane sheet, eliminating the need for gluing two chambers together without introducing air into the chamber; 2) cell numbers were assessed by using a colourimetric assay based on either cell metabolism (MTT), or on total protein mass (sulforhodamine B), eliminating the hazards of potential spills of radiolabelled cells. Both colourimetric assays gave absorbance values that were accurate and linearly correlated with cell numbers [Rubenstein et al, 1990] (data not shown).

Expression of integrin subunits on normal and malignant melanocytes by flow cytometry

All three cell lines expressed ß1, α3, α2, and α5 at levels greater than twofold over baseline (Table IX-1), however, α5 expression on normal melanocytes was not significant ($p<0.05$). Expression of α4 and ß2 was not found. Normal melanocytes expressed more ß4 than either malignant melanoma cell line, but was not significant. Amelanotic, metastatic G361 cells expressed more αv than either normal melanocytes or melanotic, metastatic SK-MEL23 cells, but was not significant. However, ß3 expression was not found on either normal melanocytes or G361 cells, and only minimally expressed on SK-MEL23 cells (less than 10% above baseline). No correlation was found between integrin subunit expression and differentiation state in each of three cell types. This is in contrast with the finding of Albelda and colleagues [1990], who indicated an increased expression of αvß3 on malignant melanoma cells *in vitro* with respect to tumour progression and metastasis.

Melanocyte adhesion to substrates

The best adhesive substrate for normal human melanocytes under normal media conditions (magnesium/calcium) was fibronectin, followed by laminin, collagen type IV and collagen type I (Figure IX-1). Blocked plastic microwells had a very low attachment (< 10%). The strength of adhesion increased as a function of time, with maximal adhesive strength at 2 hr (data not shown). Adhesion strength also decreased with decreasing incubation temperature (range, 4°C. - 25°C.).

The G361 and SK-MEL 23 metastatic melanoma cells exhibited different preferences for substrate: collagens were the best substrates, followed by laminin and fibronectin (Figures IX-2 and IX-3). Compared to normal melanocytes, amelanotic G361 cells had a similar adhesive strength on all substrates, whereas melanotic SK-MEL 23 cells had a 50% lower adhesive strength on all substrates. Since flow cytometry showed that the integrin subunit expression was greater for metastatic melanoma cell lines compared to normal nonmetastatic melanocytes, the cell-specific variation in adhesion strength cannot be explained by integrin surface distribution alone.

Effect of substrate preparation on melanocyte adhesion

No diminution of extracellular matrix protein adsorption was detected by either the addition of EDTA to the ECM solution, or by rinsing the adsorbed protein after blockade (data not shown). Since divalent cations may have affected protein adsorption or the conformational structure of extracellular matrix molecules, it was necessary to determine the effect of pretreatment of the substrates with EDTA or EGTA prior to cell adhesion. Since there was no diminution of adsorbed substrate concentration with chelator treatment, the mechanism of ECM adsorption onto plastic is independent of divalent cations.

The adhesion of cells to substrates was significantly diminished after EDTA-pretreatment, compared to the nonchelated surfaces (Table IX-2). The observed decrease in adhesion was not due to remnant chelator which might interfere with integrin function, as each well surface was extensively washed with PBS prior to performing the cell adhesion assay. Furthermore, since the same decreased adhesion was also observed with EGTA pretreatment (data not shown), calcium may be responsible for the increased adhesion to nonchelated substrates.

Divalent cation-dependent adhesion of melanocytes

Normal melanocytes bound to all substrates, but required the presence of divalent cations, since low adhesion was seen in the presence of 10 mM EDTA (Figure IX-1). Adhesion on each substrate altered when the cation species was changed. Manganese (1mM) provided the strongest adhesion, followed by magnesium/calcium, manganese/magnesium, magnesium, and calcium, in the order of decreasing adhesion strength. Similarly, both G361 and SK-MEL23 melanoma cell lines bound to all substrates, and required the presence of divalent cations (Figures IX-2 and IX-3). Removal of divalent cations by EDTA caused a very low adhesion state. Both cell lines also showed a similar order of adhesion strength, dependent on the cation species present. For G361 melanoma cells, manganese (1 mM) provided the best adhesion, followed by manganese/magnesium, magnesium/calcium, magnesium, and calcium, in order of descending adhesion (Figure IX-2). SK-MEL 23 melanoma cells exhibited different affinities for substrates in the presence of the same divalent cations (Figure IX-3). Manganese/magnesium provided the best adhesion, followed by manganese, magnesium/calcium, magnesium, and calcium, in descending order of adhesion affinity. While the magnitude of the order was different from G361 melanoma cells, SK-MEL 23 melanoma cells also demonstrated this order, regardless of substrate.

If divalent cations are truly modulating the affinity of integrins via the cation-binding sites on the extracellular domain of the α-subunit, then cations may be able to induce adhesion in a nonadherent cell line. To clarify this possibility, normally non-adherent H-69 small cell lung carcinoma cells were examined. H-69 expresses only two integrins, $\alpha3\beta1$, and $\alpha6\beta1$, and grow in suspension. H-69 did not stick to fibronectin or laminin in the presence of calcium and magnesium (Figure IX-4). However, manganese augmented adhesion significantly to all substrates. If manganese was combined with magnesium, adhesion was greatly increased to a level greater than each cations combined, implying a synergistic effect on adhesion strength.

Cations induces binding to blocked substrates, and secretion of extracellular matrix, but cells are not able to attach

All three cell lines adhered to plastic blocked with 5% nonfat milk in the presence of manganese, magnesium and their combinations, but not with calcium or magnesium alone (Figures IX-1 to IX-3). One explanation was that the blocking agent was contaminated with some extracellular matrix molecule, or a cation-dependent binding site was present on casein that permitted adhesion. However, neither 5% bovine serum albumin, 5% ovalbumin nor 0.7% lambda carageenan was capable of preventing adhesion to plastic in the presence of manganese (Table IX-3). The exception was lambda carageenan, which was a poor blocking agent in both EDTA and manganese containing conditions. Hence, the presence of manganese in the extracellular space provided some stimulus for these cells to adhere to the plastic, regardless of the blocking agent.

A second explanation was that the cells, when exposed to these cations, secreted a substrate that could bind to the blocked plastic, which could allow adhesion to occur. No extracellular matrix was detectable on adherent cells in the presence of EDTA by immunofluorescence (Figure IX-5). Laminin, but not fibronectin or collagen type IV was detected by immunofluorescence on adherent cells cultivated for one hour in the presence of either calcium, magnesium, or manganese (Table IX-4). However, examination of these same cells by flow cytometry after harvesting with EDTA did not demonstrate laminin, fibronectin, or collagen type IV on the surface of these cells. This suggests that the cells may secrete the laminin into the extracellular space, rather than retain it on the cell surface.

The three cell lines tested did not adhere to the cell-secreted laminin because the laminin did not adsorb to the blocked plastic (data not shown). Furthermore, G361 did not adhere to preadsorbed laminin in the presence of calcium (Figure IX-2). Hence, cations may cause the secretion of laminin, but this secreted substrate was unable to adsorb to the blocked plastic.

Each cell line required a threshold concentration of manganese, below which adhesion was minimal. However, minimal elevation of extracellular manganese concentrations dramatically increased cell adhesion (Figure IX-6). Decreasing adhesion was detected at manganese concentrations above 2 mM. Since manganese is toxic to cells at high concentrations [Edwards et al, 1988], the decreased adhesion may partly reflect the effect of manganese toxicity. This effect was observed regardless of the type of blocking agent used (data not shown).

Manganese activates adhesion to blocked substrates through a RGD-sensitive mechanism

Evidence for extracellular activation of integrin avidity was supported by the ability to directly inhibit cell adhesion by increasing concentrations of soluble GRGDS peptides (Figure IX-7). No decrease in cell-substrate adhesion was observed with the nonfunctional peptide, GRGES. The increased adhesion observed with manganese may, therefore, be due to the increased recognition and avidity for RGD adhesion sequences on the substrates.

In order to further explore the functional role of specific integrin subunits, blocking G361 adhesion to RGD-sensitive sites on blocked plastic was examined. The antibodies with known functional blocking abilities against $\alpha 3$, ß1 and ß4 subunits impeded adhesion greater than 40% (Table IX-5). A minor inhibition of adhesion was observed with $\alpha 2$, while no inhibition was seen with the antibodies against $\alpha 5$ and ß3 subunits. The degree of inhibition was almost complete with the anti-ß1 antibody. It may be reasonable to expect that at least one ß1 integrin family member is responsible for cell adhesion. However, the moderate inhibition observed with the anti-ß4 antibody may imply that the $\alpha 6$ß4 integrin also plays a role, despite its low expression on the cell surface as measured by flow cytometry (Table IX-1).

DISCUSSION

Normal and malignant melanocytes with different cellular differentiation markers have common integrin subunits but different degrees of expression

We examined three melanocyte cell types at different stages of differentiation. One cell type was the normal human epidermal melanocyte with no metastatic ability, and the other two types were the human metastatic melanoma cell lines G361 and SK-MEL23 with different stages of cell differentiation. Houghton and his colleagues [1987] outlined morphologic features of cultured melanoma cells that define early or late stages of differentiation. According to his classification, G361 melanoma cells show early-stage differentiation markers, with an epithelioid morphology, an absence of pigment, and low tyrosinase activity. SK-MEL23 melanoma cells express late-stage differentiation markers, with a spindle-shaped or dendritic cell morphology, and a high degrees of pigment expression and tyrosinase activity. Our recent study has confirmed the same degree of differentiation stages on these two melanoma cells [Hara et al, 1994].

Despite the differences in the cellular differentiation, no different patterns were noted in integrin expression. Several integrin subunits with overlapping substrate affinities were expressed on both normal and malignant melanocytes. No cells expressed a unique subunit, but the degree of expression between cell types was quite distinct. Neither the integrin αv or ß3 was highly expressed, nor was a pattern of increasing expression with decreasing differentiation noted. While this appears contrary to the findings of Albelda and colleagues [1990], it could be reconciled by the heterogeneity between tumour cell lines, as was also found by Albelda. However, we concur that no clear pattern emerges that links degree of integrin expression with substrate adhesion, implying a more complex association between the integrins and cell adhesion.

Divalent cations modulate integrin-dependent cell adhesion

This study indicated that normal nonmetastatic and malignant metastatic tumour melanocytes adhere to extracellular matrices in a divalent cation-dependent manner. For any one substrate, cells preferentially bind in the presence of manganese, followed by magnesium, then calcium. These cations induce secretion of laminin into the extracellular space, but laminin is not expressed on the cell surface. The secreted extracellular matrix was not capable of binding to the blocked substrate surface.

The cation modulating mechanism of integrin adhesion involves a noncovalent association with binding sites on the extracellular region of integrins. Amino acid sequence analysis of the α-subunit reveals three to four domains in the extracellular region that have a high homology to the "EF-hand" sequence, a cation-binding region found in cation-dependent kinases [Edwards et al, 1988; Dransfield et al, 1990; Loftus et al, 1990]. The binding site forms a pocket lined by oxygen-containing amino acid side groups that form hydrogen bonding coordination sites. The integrin binding sites have five of the six coordination sites [Loftus et al, 1990]. The sixth site comes from either a domain on the ß-subunit, or from the ligand/ß-subunit complex formed during interaction with the ligand domain. Different divalent cations have different ionic radii, which may either expand or compress the binding pocket, and cause conformational changes in either integrin subunit [Grzesiak et al, 1992, Declerq et al, 1991]. This study suggests that calcium provides a favourable adhesive conformation to the integrin, but the smaller ionic radius of manganese provides a superior adhesive conformation. The much smaller ionic radius of magnesium may distort the molecule to such a degree that ligand affinity is diminished.

Matsumoto and Hemler [1993] have recently shown that conservative substitution of one of the coordination site amino acids on α4ß1 by site-directed mutagenesis can interfere with cation binding in that site, and block adhesion to specific ligand domains. Futhermore, they have shown that interference with each of the different cation-binding sites causes alterations in affinity for specific ligands. Thus, control of cation occupancy of each binding site could determine ligand affinity. If one conservatively considers that three cation-binding sites may be occupied by only three different extracellular cations, then the receptor has 27 different activation states. It is not known how this diversity can specifically alter the conformation of the integrin subunits, and how these conformational changes are translated into intracellular signalling and organization of the cytoskeleton.

One explanation would be that a weak adhesion state occurs when binding sites are occupied by a cation species which produces a conformation that binds to the actin cytoskeletal complex with low affinity. Conversely, occupancy by another cation that produces a conformation that can bind the actin cytoskeletal complex with high affinity, and would produce a strong adhesion state. Alternatively, since cations are used by other systems, like membrane ion pumps, a particular cation may alter integrin-mediated activation of adhesion-related kinases, which may further be affected by different concentrations of submembranic cations. That integrin activation affects cytoplasmic ion concentrations has been shown by Schwartz with the demonstration of pH increase by fibronectin binding with $\alpha5\beta1$ [Schwartz et al, 1991].

Divalent cations also modulate integrin-independent cell adhesion

Adhesion is found on "nonadherent, blocked" substrates in the presence of manganese. This is independent of the nature of the blocking agent, and is in direct proportion with the manganese concentration. While it is not competitively inhibited by other divalent cations, it can be inhibited in a dose-dependent manner by soluble RGD peptide, and by functional anti-$\beta1$, $\alpha2$, $\alpha3$, and $\beta4$ antibodies, suggesting that integrins are involved at some stage of the adhesion complex formation.

Cell adhesion to blocked substrates in the presence of manganese suggest an alternative adhesion mechanism. Grinnell and Backman [1991] noted that cells could adhere to nonadherent surfaces in the presence of manganese, and proposed an integrin-independent mechanism, based on conformation of the integrin into a constitutively activated state. Our results cannot refute this observation. Since soluble RGD peptides can block adhesion, this blockade implies that integrins are necessary for, at least, a part of the adhesion cascade. Blockade of integrin function may prevent the next phase of attachment from occurring, leading to a less robust adhesion complex.

The disruption may occur at the extracellular region, by interfering with complex formation, or on the cytoplasmic region, by disrupting the cytoskeletal association with the bound integrins.

The role of divalent cations in the adhesion cascade

How can cells use divalent cations for adhesion? One possibility might be the following cascade of events, based upon previous studies and our own findings. Cells express a variety of cell surface receptors, some of which are bound to ligand [Ruoslahti and Pierschbacher, 1987]. The bound receptors are activated, and interact with a cytoplasmic adhesion complex [Masumoto and Hemler, 1993]. Other receptors are expressed on the surface, but are not bound. These receptors may be in a variable activation state [Masumoto and Hemler, 1993]. A change in the concentration of extracellular cations causes an alteration in the occupancy state of the cation-binding sites on the α-integrin subunit [Edwards et al, 1988; Grzesiak et al, 1992; Dransfield et al, 1990; Loftus et al, 1990; Declerq et al, 1991; Masumoto and Hemler, 1993]. This causes a conformational change from an inactivated to an activated form, as if the ligand was bound [Edwards et al, 1988; Dransfield et al, 1990; Loftus et al, 1990; Masumoto and Hemler, 1993]. The cell, recognizing this activated state on the cytoplasmic side, initiates a series of events, including cytoplasmic adhesion complex formation, and other signals to intracellular processes [Masumoto and Hemler, 1993]. Activation of integrins may, in turn, activate other unknown non-integrin adhesion receptors, reinforcing adhesion to other substrate ligands. Thus, the observed increase in adhesion in our study of normal and malignant melanocytes with different stages of cellular differentiation is the result of more advanced adhesion processes that can be triggered by integrin activation, but do not exclusively require integrins for maintenance of adhesion complex formation. Involvement of the cytoskeleton appears necessary, and would probably require other intracellular signal transduction pathways as well. The nature of this signalling, and their regulation is not known.

Secretion of extracellular matrix by cells in response to cations hints at a possible paracrine function for cells. Shifts in extracellular cation concentrations may occur in periods of stress, such as cell death, trauma, or during morphogenesis [Grzesiak et al, 1992]. The change in cation concentration may provide a first signal for altered behaviour. The cell may secrete a preformed extracellular matrix into the environment as a modulating 'second' signal, which would be detected by cellular integrins. While the secreted extracellular matrix components did not bind to the substrate in our assay, they may interact with the complex environment found *in vivo*. Furthermore, the enhanced avidity of cation-bound integrins for substrates may rapidly amplify the initial signal (within minutes).

In conclusion, our modified vertical centrifugal detachment assay has shown that both normal and malignant melanocytes adhere to extracellular matrix molecules found in the basement membrane of normal skin and healing wounds in a divalent cation-dependent manner. Integrins are found to be responsible in part for this adhesion. The integrins can be rapidly activated by small alterations in the extracellular cation concentration surrounding the integrins, which is reflected in cell adhesion. Since the activation occurs over a very short time period, this allows cells to rapidly respond to changes in their microenvironment. This may be exploited by malignant melanocytes to permit cell motility, migration and metastasis through matrices, and the rapidity of modulation may permit these malignant cells to respond to a dynamic, hostile environment, such as that found in a blood vessel after extravasation.

TABLE IX-1: Median fluorescence intensities for integrin expression by flow cytometry for human melanocytes

Integrin Subunit[a]	Median Fluorescence Intensity[b]		
	Normal Melanocytes	G361[d]	SK-MEL 23[d]
Negative Control[c]	37.0	48.0	33.25
α2	84.0[e]	230.0[e]	185.4[e]
α3	189.9[e]	250.0[e]	188.5[e]
α4	37.0	48.0	34.0
α5	63.0	82.0[e]	87.4[e]
αv	45.0	80.0	36.3
β1	340.0[e]	400.0[e]	600.0[e]
β2	37.5	48.2	33.7
β3	37.0	48.1	38.5
β4	55.0	50.0	36.5

a: as identified by the appropriate monoclonal antibody.

b: as calculated by flow cytometer software [LYSIS II, Becton-Dickenson] (arbitrary units).

c: labelled with phycoerythrin-conjugated secondary antibody alone.

d: malignant human melanocytes (human melanoma cell lines).

e: statistically significant ($p < 0.05$)

TABLE IX-2: Effect of substrate pretreatment with 10 mM EDTA on G361 melanoma cell adhesion.

SUBSTRATE	PERCENT ADHESION[a]	
	CONTROL	EDTA (10 mM)
Plastic	8.2 ± 1.2[c]	4.5 ± 0.8
Fibronectin[b]	65.0 ± 1.6	61.0 ± 1.3
Laminin	52.5 ± 1.0	46.3 ± 1.5
Collagen Type I	59.2 ± 2.1	50.5 ± 1.6
Collagen Type IV	57.3 ± 1.7	51.6 ± 1.8

a: calculated value (adherent fraction abs - blank control abs)/[(adherent fraction abs - blank control abs) + (nonadherent fraction abs - blank control abs)] x 100

b: adsorption concentration 10 μg/ml

c: ± standard deviation

$p < 0.0005$ for all values

TABLE IX-3: Effect of blocking agents on cell adhesion of G361 melanoma cells to plastic

BLOCKING AGENT[a]	PERCENT ADHESION[b]	
	EDTA (10 mM)	MANGANESE (1 mM)
None	22.6 ± 7.0[c]	48.6 ± 3.0
5% Nonfat Milk (w/v)	4.3 ± 3.0	48.2 ± 4.0
5% bovine serum albumin	3.9 ± 2.0	35.0 ± 5.0
5% ovalbumin	3.5 ± 1.0	37.5 ± 1.5
0.7% carageenan	40.0 ± 3.0	42.0 ± 3.5

a: Incubation time of one hour at 37°C.

b: Values calculated as in Table 2.

c: ± standard deviation

$p < 0.0005$ for all values

TABLE IX-4: Effect of divalent cations on expression of fibronectin, laminin and collagen type IV by G361 cells on blocked plastic[a].

CATION (1 mM)	AVERAGE OPTICAL DENSITY[b]			
	PLASTIC	FIBRONECTIN[c]	LAMININ	COLLAGEN IV
EDTA	0.041 ± 0.003	0.036 ± 0.004	0.055 ± 0.004	0.047 ± 0.003
Calcium	0.034 ± 0.005	0.035 ± 0.005	0.121 ± 0.006[d]	0.033 ± 0.004
Magnesium	0.036 ± 0.005	0.047 ± 0.003	0.101 ± 0.005[d]	0.034 ± 0.005
Manganese	0.040 ± 0.004	0.035 ± 0.005	0.105 ± 0.006[d]	0.039 ± 0.006

a: Plastic blocked with 5% (w/v) nonfat milk powder/PBS.

b: Optical density scale: 0.0 = white; 1.0 = black.2

c: Substrate coating concentration 10 µg/ml.

d: $p < 0.0005$

TABLE IX-5: Effect of specific antibodies in blocking of cell adhesion of G 361 melanoma cell adhesion on blocked plastic[a]

ANTIBODY[b]	PERCENT OF CONTROL ADHESION[c]
Control[d]	100.0
α2	83.4
α3	63.5
α5	96.0
ß1	3.0
ß3	95.0
ß4	58.6

a: Plastic blocked with 5% (w/v) nonfat milk powder/PBS.

b: Protein concentration of purified antibody: 2 μg/ml

c: Calculated value: (Percent adhesion with antibody / Percent adhesion without antibody) x 100.

d: Control had no antibody added to incubation solution.

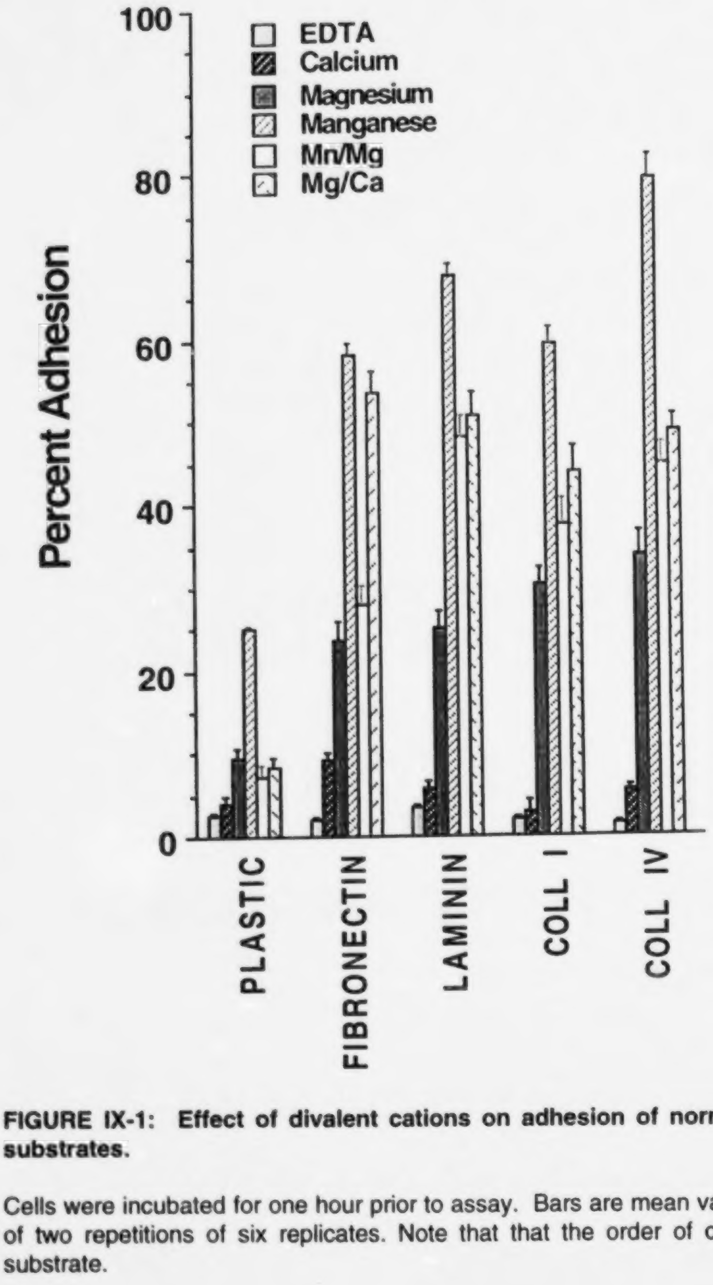

FIGURE IX-1: Effect of divalent cations on adhesion of normal human melanocytes to substrates.

Cells were incubated for one hour prior to assay. Bars are mean values and standard deviations of two repetitions of six replicates. Note that that the order of cations is the same for each substrate.

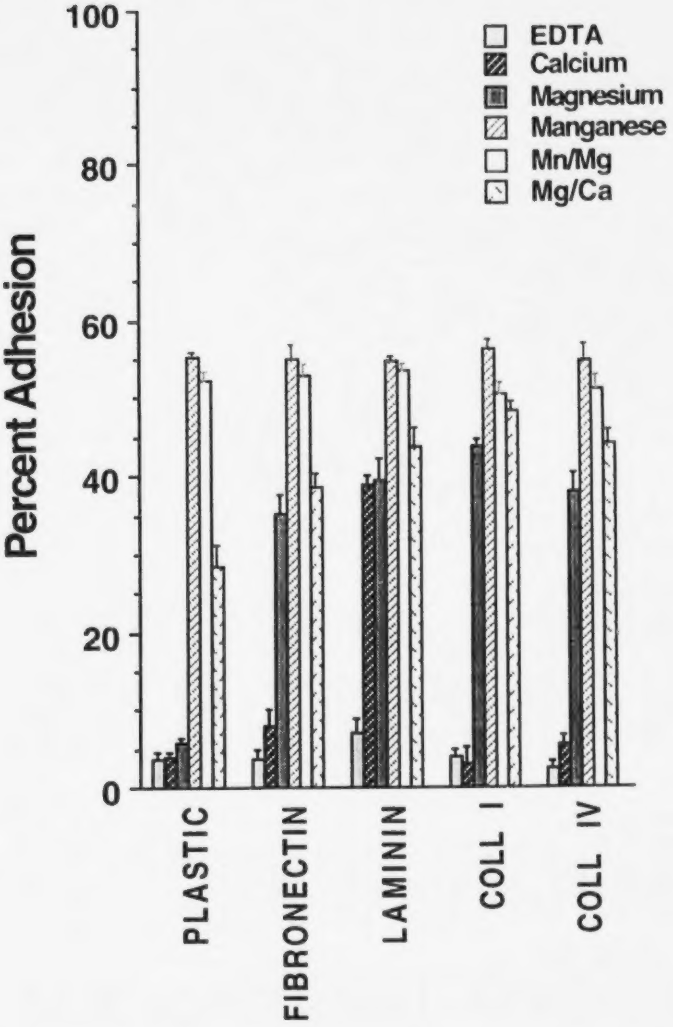

FIGURE IX-2: Effect of divalent cations on adhesion of malignant melanoma cell line G361 to substrates.

Cells were incubated for one hour prior to assay. Bars are mean values and standard deviations of two repetitions of six replicates. Note that that the order of cations is the same for each substrate.

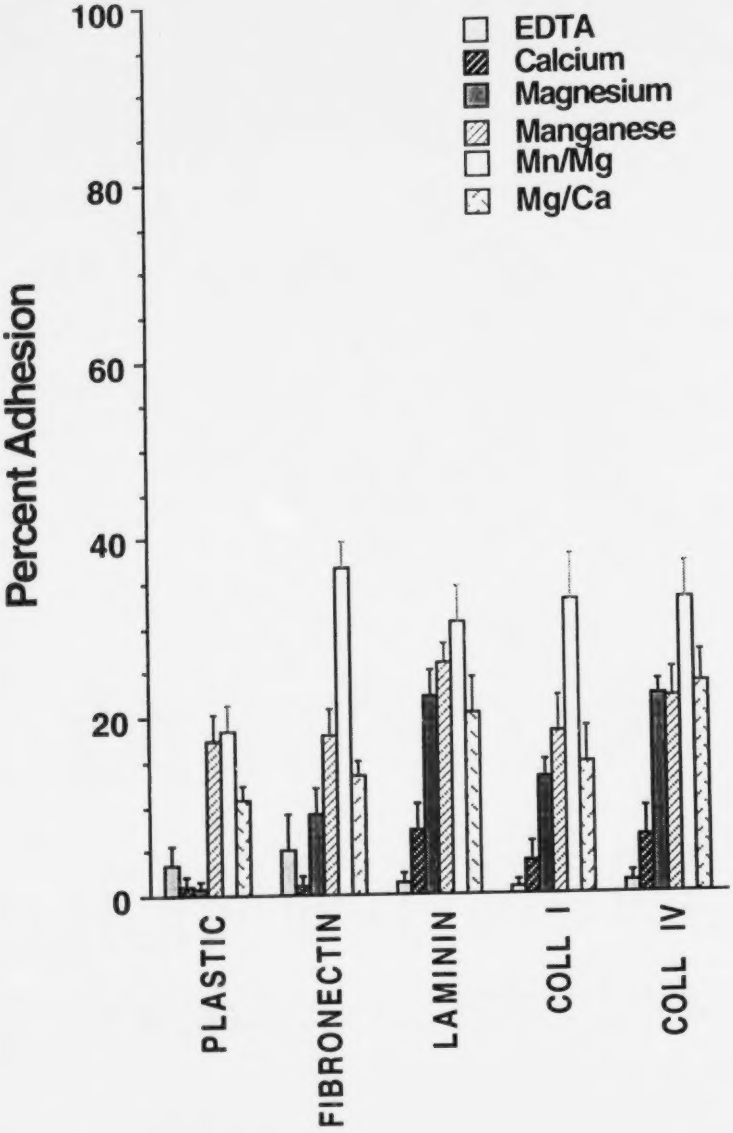

FIGURE IX-3: Effect of divalent cations on adhesion of malignant melanoma cell line SK-MEL23 to substrates.

Cells were incubated for one hour prior to assay. Bars are mean values and standard deviations of two repetitions of six replicates. Note that that the order of cations is the same for each substrate.

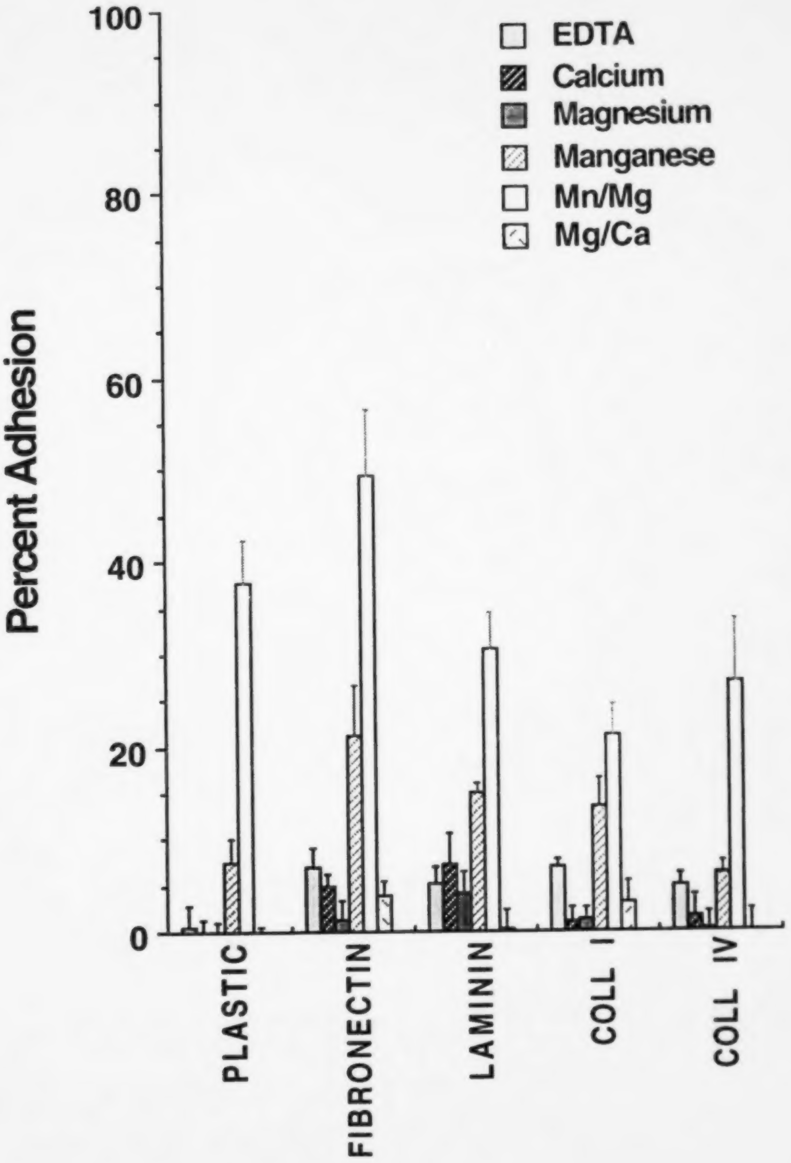

FIGURE IX-4: Effect of divalent cations on adhesion of small cell lung carcinoma cell line H-69 to substrates.

Cells were incubated for one hour prior to assay. Bars are mean values and standard deviations of two repetitions of six replicates. Note that that the order of cations is the same for each substrate in the adherent states.

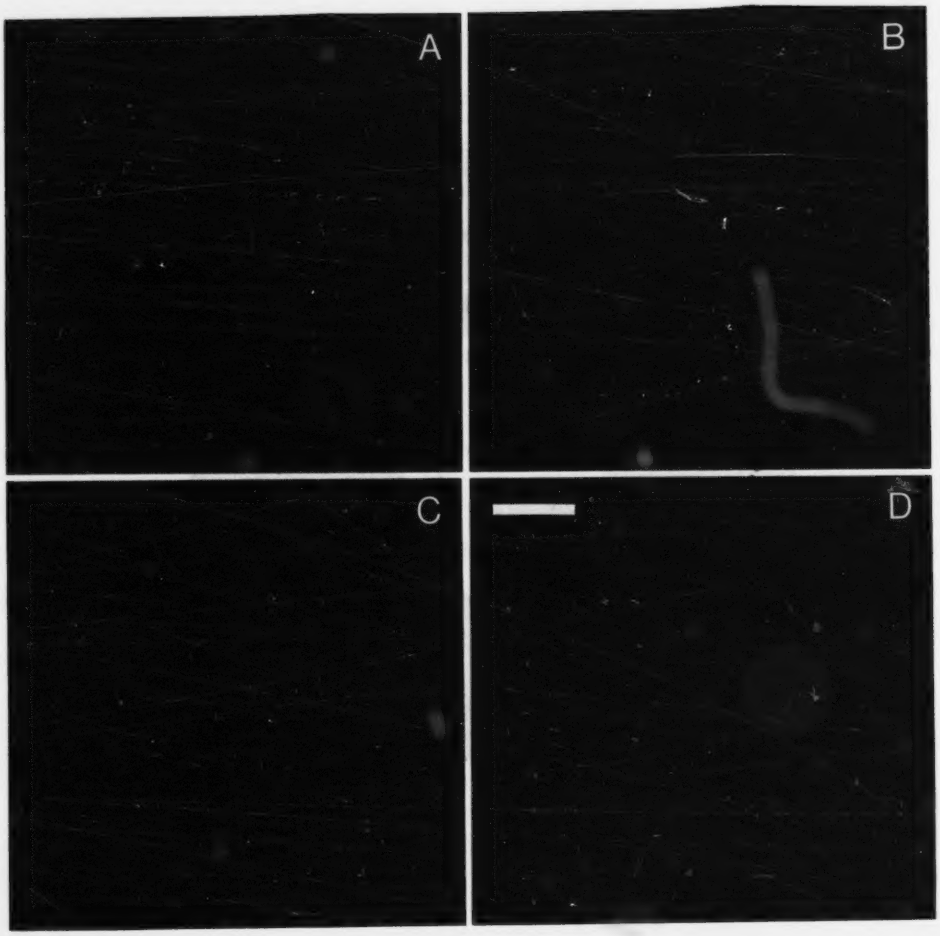

FIGURE IX-5: Effect of divalent cations on expression of laminin by G361 cells on blocked plastic.

Immunofluorescence of anti-laminin antibody under a: EDTA; b: Calcium (1 mM); c: Magnesium (1 mM); d: Manganese (1 mM). Note that only laminin is detected on cells exposed to divalent cations. Bar represents 20 μm.

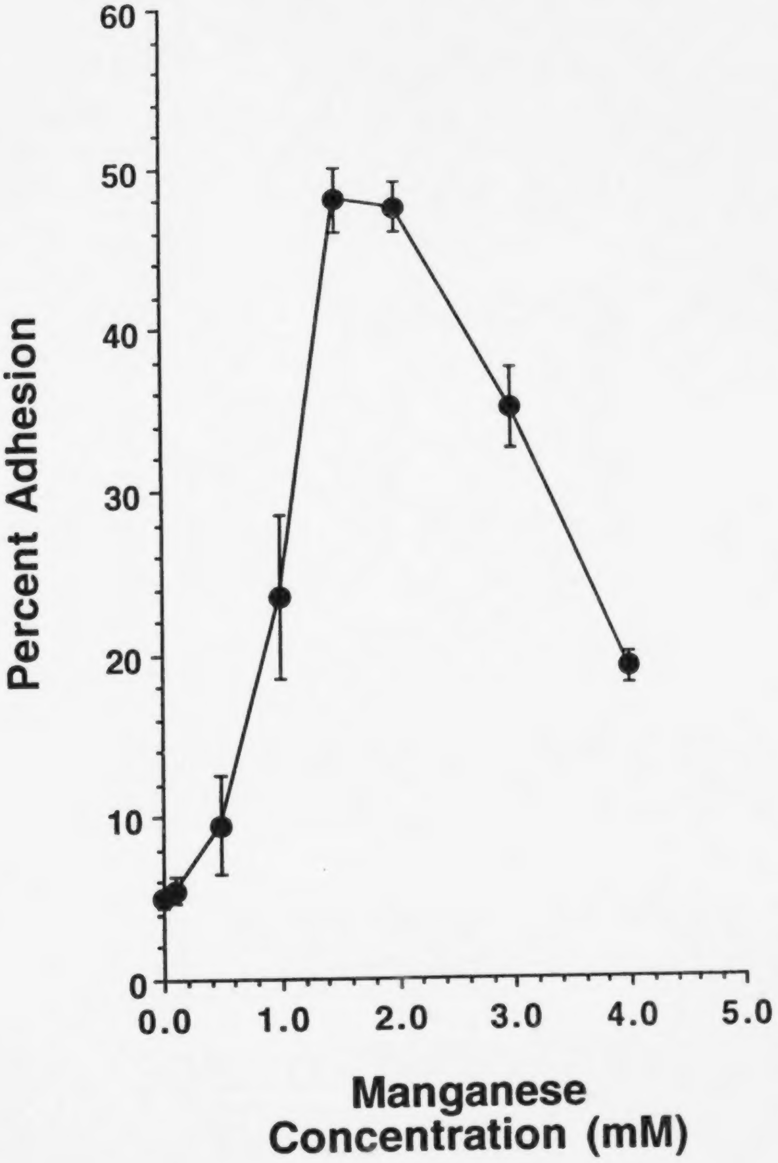

FIGURE IX-6: Effect of increasing manganese concentration on G 361 adhesion to blocked plastic substrate.

Note that adhesion decreases at concentrations above 2 mM.

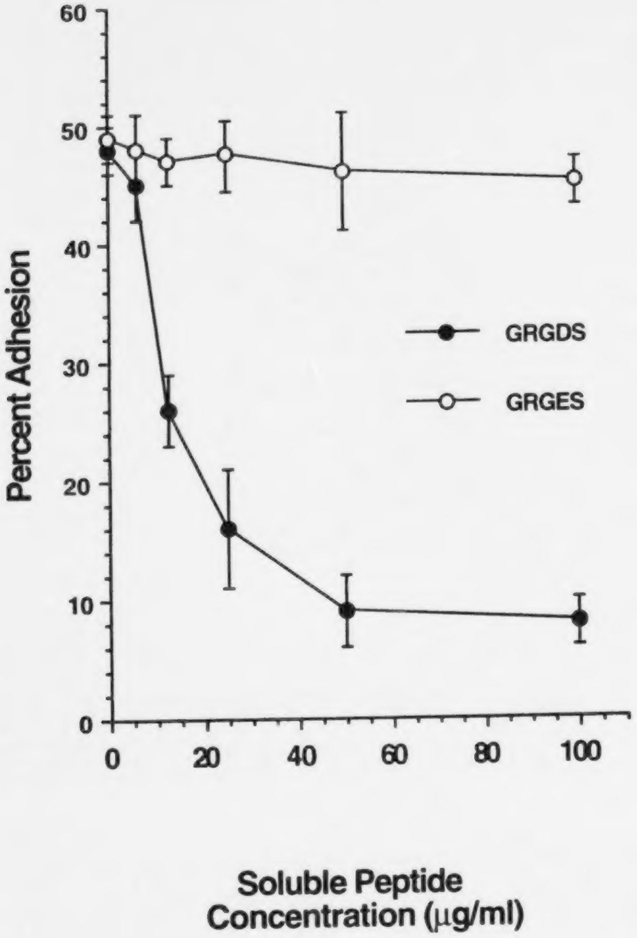

FIGURE IX-7: Effect of soluble GRGDS and GRGES peptide on G 361 adhesion to blocked plastic.

Values calculated from 200 x g.

BIBLIOGRAPHY

Albelda SM, SA Mette, DE Elder, R Stewart, L Damjanovich, M Herlyn, C Buck. Integrin distribution in malignant melanoma: association of the ß3 subunit with tumor progression. Cancer Res. 50:6757-64, 1990.

Clark WH, DE Elder, D Guerry, MN Epstein IV, MH Greene, M Van Horn. "A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma." Hum. Path. 15:1147-1165, 1984.

Declerq J, B Tinant, J Parello, J Rambaud. Ionic intereactions with parvalbumins. Crystal structure determination of pike 4.10 parvalbumin in four different ionic environments. J. Mol. Biol. 220:1017-1039, 1991.

Dransfield I, A-M Buckle, N Hogg. Early events of the immune response mediated by leukocyte integrins. Immunol. Rev. 114:29-44, 1990.

Edwards JG, H Hameed, G Campbell. Induction of fibroblast spreading by Mn^{2+}: a possible role for unusual binding sites for divalent cations in receptors for proteins containing Arg-Gly-Asp. J. Cell Sci. 89:507-513, 1988.

Eisinger M, Marko O. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc Natl Acad Sci USA 79:2018-2022, 1982.

Grinnell F, R Backman. Role of integrin receptors in manganese-dependent BHK cell spreading on albumin-coated substrata. Exp.Cell Res, 195:218-223, 1991.

Grzesiak JJ, GE Davis, D Kirchhofer, MD Pierschbacher. Regulation of α2ß1-mediated fibroblast migration on type I collagen by shifts in the concentrations of extracellular Mg^{+2} and Ca^{+2}. J.Cell Biol. 117:1109-1117, 1992.

Halaban R, FD Alfano. Selective elimination of fibroblasts from cultures of normal human melanocytes. In Vitro. 20:447-450, 1984.

Hara H MH Lee, H Chen, D Luo, K Jimbow. Role of gene expression and protein synthesis of tyrosinase, TRP-1, Lamp-1, and CD 63 in UVB-induced melanogenesis in human melanomas. J. Invest. Dermatol. 102:495-500, 1994.

Houghton AN, FX Real, LJ Davis, C Cordon-Cardo, LJ Old. Phenotypic heterogeneity of melanoma: relation to the differentiation program of melanoma cells. J. Exp. Med. 164:812-829, 1987.

Loftus JC, TE O'Toole, EF Plow, A Glass, AL Frelinger III, MH Ginsberg. A ß3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. Science, 249:915-8, 1990.

Lotz MM, CA Burdsal, HP Erickson, DR McClay. Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. J.Cell Biol, 109:1795-1805, 1989.

Masumoto A, ME Hemler. Mutation of putative divalent cation sites in the $\alpha4$ subunit of the integrin VLA-4: distinct effects on adhesion to CS1/fibronectin, VCAM-1, and Invasin. J. Cell Biol. 123:245-253, 1993.

McClay DR, GM Wessel, RB Marchase. Intercellular recognition: quantitation of initial binding events. Proc.Natl.Acad.Sci, USA. 78:4975-4979, 1981.

Rubenstein LV, RH Shoemaker, KD Paull, RM Simon, S Tosini, P Skehan, DA Scudiero, A Monks, MR Boyd. Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J. Natl. Cancer Inst. 82:1113-1118.

Ruoslahti E, MD Pierschbacher. New perspectives in cell adhesion: RGD and integrins. Science. 238:491-497, 1987.

Schwartz MA, C Lechene, DE Ingber. Insoluble fibronectin activates the Na/H antiporter by clustering and immobilizing integrin $\alpha5\beta1$, independent of cell shape. Proc.Natl. Acad. Sci. (USA) 88:7849-7853, 1991.

Skehan P, R Storeng, D Scudiero, A Monks, J McMahon, D Vistica, JT Warren, H Bokesch, S

Kenney, MR Boyd. "New colorimetric cytotoxicity assay for anticancer-drug screening." J. Natl. Cancer Inst. 82:1107-1112, 1990.

Sofroniew MV, Schrell U: Long-term storage and regular repeated use of diluted antisera in glass staining jars for increased sensitivity, reproducibility, and convenience of single- and two-color light microscopic immunocytochemistry. J Histochem Cytochem 30:504-511, 1982.